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University of Kent
Faculty of Sciences
School of Sport and Exercise Science

Exercise induced inflammation,
and the effect of omega-3 polyunsaturated
fatty acids on physiological variables
associated with endurance performance

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A thesis submitted for the degree of
Doctor of Philosophy

March 2018

ABSTRACT

The purpose of this thesis was to investigate the effect of a dietary intervention designed to increase tissue incorporation of omega 3 polyunsaturated fatty acids (*n*-3 PUFA) on oxygen consumption during submaximal exercise, and to investigate the acute inflammatory cytokine response to different intensities of cycling in trained men.

Study 1. The cytokine response to two cycling modalities: 1) pre-loaded performance trial and 2) high intensity interval trial.

This study investigated the effect of two cycling protocols on plasma concentrations of pro-inflammatory and anti-inflammatory cytokines. Fourteen moderately trained healthy males completed two cycling protocols 1 week apart, 1) a 1-hour pre-loaded cycling time trial with the first 45 minutes corresponding ~70% of maximal work load and 2), 12 x 1 minute high intensity intervals corresponding to 100% of maximal workload (workload was based on an incremental test to exhaustion). Blood samples were obtained from the antecubital vein pre and immediately post each exercise trial, for the determination of plasma cytokine concentrations. Additional saliva samples were collected at the same time points to validate their relationship and agreement with plasma samples for measurement of IL-1 β . Results: IL-6 increased more markedly following the pre-loaded time trial (mean \pm standard deviation), 2.56 ± 3.30 pg./ ml to 6.68 ± 4.48 pg./ ml respectively (a 161% increase) compared to the interval trial 2.16 ± 2.05 pg./ ml to 2.95 ± 2.24 pg./ ml (a 37% increase) (time * trial interaction $p = 0.015$). IL-4 and MCP-1 concentrations increased, and VEGF decreased following both trials combined (main effect of time IL-4, $p = 0.013$, MCP-1, $p = 0.002$, VEGF, $p = 0.016$). No changes were observed

in other cytokine concentrations. A significant correlation between plasma and saliva IL-1 β concentration was only observed in samples obtained post the time trial ($r = 0.807$, $p = 0.009$), expressed as concentration: osmolality, $r = 0.781$, $p = 0.013$). In conclusion, a 1-hour pre-loaded cycling trial resulted in a greater increase in IL-6 compared with 12 x 1min interval trial. Acute post exercise increases in IL-4 and MCP-1, and a decrease in VEGF were observed post both trials on average. Furthermore, the relationship between plasma and saliva for the measurement of IL-1 β concentration was inconsistent and lacked agreement. Saliva did not prove to be a valid alternative method of measurement to blood plasma for the measurement of IL-1 β .

Study 2. Validation of the use of a fingertip capillary dry blood spot method to determine percentage incorporation of n-3 PUFA and n6 into erythrocyte cell membranes.

This study investigated the relationship and agreement between dry blood spot samples (DBS), and isolated erythrocytes (from venepuncture whole blood samples), for the measurement of percentage PUFA status in 10-trained cyclists. Blood samples were collected on three occasions over an 8-week period (baseline, at 4 weeks and at 8 weeks) to determine percentage tissue incorporation of total n -6 PUFA, AA, LA, total n -3 PUFA, EPA and DHA. On each occasion, two sample types were obtained from each participant. 1), isolated erythrocytes from whole blood taken from the participant's antecubital vein. 2), a whole blood fingertip capillary sample spotted on to a Guthrie absorbent card pre-soaked in butylated hydroxytoluene. The n -3 PUFA and n -6 PUFA content of both samples was determined by gas chromatography and mass spectrometry. Results: Isolated erythrocyte samples had a significantly higher percentage content of EPA, AA, total

n-6, EPA + DHA and *n*-6/ *n*-3 PUFA and a lower content of ALA when compared to fingertip capillary DBS samples. No differences between methods were observed for DHA and total *n*-3 PUFA values. Linear regression showed there was a significant correlation (*R*) between the two types of measurement for EPA ($R^2 = 0.64$, $p = 0.005$) but not for other fatty acids. Bland and Altman analysis showed moderate agreement between methods for EPA (mean difference = 1.12% with 95% limits of agreement -1.43% – 3.67%). In conclusion, the current study suggests fingertip capillary DBS sampling is a valid method for determining the EPA status of individuals but not for other *n*-3, *n*-6 PUFAs.

Study 3. The effect of an 8-week high n-3 PUFA and low n-6 PUFA (H3-L6) dietary intervention, on erythrocyte fatty acid incorporation, and its effect on the oxygen cost of submaximal exercise and $\dot{V}O_{2\text{ peak}}$ in trained cyclists.

This study investigated the effect of an 8-week dietary intervention (designed to increase *n*-3 PUFA and reduce *n*-6 PUFA (to less than 2.5% of average daily kcal intake)) on $\dot{V}O_{2\text{ peak}}$ and oxygen consumption ($\dot{V}O_2$) during submaximal exercise. 14-trained male cyclists were randomly assigned to two independent groups. The experimental group received 8 x 1g capsules per day of *n*-3 PUFA (2600mg EPA and 1800mg DHA), and followed a dietary intervention designed to reduce *n*-6 PUFA intake (H3-L6). The placebo group received 8 x 1g capsules per day of organic soy oil and a pseudo dietary intervention that maintained their habitual daily *n*-6 PUFA intake. Blood samples were obtained from the participant's antecubital vein, pre supplementation, at week 4 and at week 8, for the analysis of erythrocyte incorporation of *n*-3 PUFA and *n*-6 PUFA. Pre and post the dietary intervention, participants completed 4 x 7mins submaximal cycling on a Lode

ergometer at four discontinuous incremental power outputs. Steady state oxygen consumption ($\dot{V}O_2$), tissue oxygen saturation index (TSI%), deoxygenated haemoglobin (deoxy-HHb), heart rate (HR bpm), blood pressure (BP mmHg), and rating of perceived exertion (RPE), were assessed during each cycling bout. Following a 15-minute rest, participants performed an incremental cycling ramp test to exhaustion (intensity increasing by 30 W·min⁻¹) for the determination of $\dot{V}O_{2\text{ peak}}$ (L/min). Results: H3-L6 significantly increased erythrocyte % content of total *n*-3 PUFA, EPA and DHA compared to the placebo group over the 8 week intervention (time * group interaction, total *n*-3 PUFA, $p = <0.001$, EPA, $p = <0.001$, DHA, $p = 0.001$), and significantly reduced total *n*-6 PUFA, LA and AA (time * group interaction, total *n*-6 PUFA, $p = <0.001$, LA, $p = 0.004$, AA, $p = <0.001$). There were no differences in $\dot{V}O_{2\text{ peak}}$ (group * time, $p = 0.56$). During steady state submaximal cycling, there were no differences in oxygen consumption $\dot{V}O_2$ L/min (group * time * power level, $p = 0.49$), TSI% ($p = 0.43$) or deoxy-HHb ($p = 0.56$). Furthermore, no changes were observed in HR, BP, MAP or RPE. In conclusion, despite the dietary intervention successfully increasing erythrocyte *n*-3 PUFA percentage and reducing *n*-6 PUFA incorporation over an 8-week intervention period, there was no effect on oxygen consumption during submaximal exercise or $\dot{V}O_{2\text{ peak}}$ performance.

In summary, this thesis provides evidence that IL-6 increases more markedly in response to a 1 hour pre-loaded cycling time trial compared to 12 x 1 minute intervals. For both cycling trials combined, there was an increase in anti-inflammatory IL-4 and MCP-1, and a decrease in VEGF. Furthermore, saliva did not prove to be a valid alternate measurement method to blood plasma for the measurement of IL-1 β .

This thesis demonstrated that an 8-week H3-L6 dietary intervention successfully increased erythrocyte incorporation of *n*-3 PUFA and reduced *n*-6 PUFA. However, the intervention failed to induce significant alterations in oxygen consumption during submaximal exercise or $\dot{V}O_{2\text{ peak}}$ performance. In addition, when quantifying an individual's *n*-3 PUFA and *n*-6 PUFA status, fingertip capillary DBS sampling proved to be a valid method for the measurement of EPA but not for other fatty acids. Therefore, the DBS method was not considered a suitable replacement for the isolated erythrocytes method when quantifying an individual's *n*-3 PUFA and *n*-6 PUFA status.

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I would like to acknowledge Nordic Naturals for providing the omega 3 supplement used in this thesis.

DECLARATION

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

Signed: L. Hale

Date: 27/ 03/ 18

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALA	alpha-linoleic acid
ALA	alpha
ANOVA	Analysis of variance acid fibroblast growth factor-basic
bFGF	Fibroblast growth factor-basic
BHT	Butylated hydroxytoluene
BP	Blood pressure
Ca ²⁺	Calcium
CHD	Coronary heart disease
CV	Cardiovascular
DBS	Dried blood spot
DHA	Docosahexaenoic acid I acids
EDTA	Ethylene diamine tetra acetic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FFQ	Food frequency questionnaire
FO	Fish oil
GC	Gas chromatography
HR	Heart rate
ICC	Intra-class correlation
IFN- γ	Interferon gamma
IL-10	Interleukin 10
IL-1 α	Interleukin 1 alpha
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
INT	Interval
LA	Linoleic acid
LC	Long Chain
LCPUFA	Long chain polyunsaturated fatty acids
MAP	Mean arterial pressure

MCP-1	Monocyte chemoattractant protein-1
MUFA	Monounsaturated fatty acids
<i>n</i> -3 PUFA	Omega 3
<i>n</i> -6 PUFA	Omega 6
NIRS	Near Infrared spectroscopy
NO	Nitric oxide
O ₂	Oxygen
PBMC	Peripheral blood mononuclear cell
SO	Soy oil
PBS	Phosphate buffered saline
PIGF	Placenta growth factor
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
RM	Repetition max
sFIT-1	Soluble fms-like tyrosine kinase-1
SD	Standard deviation
TNF- α	Tumour necrosis factor alpha
TSI%	Tissue saturation index (percentage)
TT	Time trial
TTE	Time to exhaustion
VEGF	Vascular endothelial growth factor
$\dot{V}O_2$	Volume of oxygen
$\dot{V}O_{2\text{ max}}$	Volume of maximal oxygen consumption
GLU	Glucose
LAC	Lactate

CHAPTER 1 INTRODUCTION

Due to the physiological demands of training and competition, athletes are constantly seeking effective dietary interventions, food supplements and ergogenic aids to improve their performance and recovery. Many athletes consume supplements daily with focus on a number of possible benefits including, delaying fatigue, maximising metabolic capacity, improving lean mass, protection for immune function, to aid recovery and maintain good health. Oily fish and fish oil supplements contain long chain *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:6 *n*-3). Long chain *n*-3 PUFAs are historically recognised for their role in growth and development (Simopoulos, 2007), in the treatment and prevention of cardiovascular disease (Marchioli et al., 2002), and for the management of hypertension, diabetes and autoimmune conditions such as rheumatoid arthritis (Simopoulos, 1999).

The consumption of *n*-3 EPA and DHA PUFA, results in the incorporation of *n*-3 PUFA into various human tissue cells in a dose response manner (Institute of Medicine, 2005., cited in European Food Safety Authority, Scientific opinion on the tolerable upper intake level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), 2012). The predominant fatty acid usually found in the tissue cell membranes of humans consuming a diet high in omega 6 fatty acids (*n*-6 FA) is arachidonic acid (AA; 20:4 *n*-6). A common explanation for the benefits of *n*-3 in the human diet is its modulatory effect on the production of inflammatory mediators (Simopoulos, 2002).

When the human diet is supplemented with EPA and DHA, cellular incorporation of *n*-3 PUFA is at the expense of AA. EPA competes with AA, and inhibits AA eicosanoid synthesis derived from the cyclooxygenase-2 and 5-lipoxygenase pathways. This reduces the production of inflammatory mediators from inflammatory cells (Mickleborough et al, 2009). This leads to a reduction in the production of prostaglandin E₂ metabolites, which cause platelet aggregation,

vasoconstriction, and induce inflammation (Mickleborough, 2008., Weylandt et al, 2012). Recent studies have identified that *n*-3 PUFAs are responsible for the production of anti-inflammatory mediators known as protectins, resolvins and maresins (Chapkin, et al, 2009). It is possible that the typical modern western diet, rich in *n*-6 PUFA, shifts the body's physiological state into one that is pro-thrombotic & pro-aggregatory resulting in increased blood viscosity, vasospasm, and vasoconstriction (Simopoulos, 2007). This may increase risk factors for disease states such as cardiovascular disease (Mozaffarian & Wu, 2011), cardiac arrhythmia (Endo & Arita, 2016) and can exacerbate autoimmune/ inflammatory diseases such as rheumatoid arthritis and asthma (Mickleborough 2013).

The hemodynamic influence of EPA and DHA supplementation in the health setting was first evaluated in the 1970's. Bang and Dyerberg carried out several pioneering pilot studies on Greenland Eskimos that demonstrated that a diet high in *n*-3 PUFA's was linked to low rates of coronary heart disease. This was theorised to be due to EPA consumption and it's antithrombotic, and cholesterol lowering effect (Bang & Dyerberg, 1980). These early studies created a new focus on the importance of *n*-3 PUFA's in the health setting, specifically the potential cardiovascular benefits of enriching the diet with EPA and DHA (Dyerberg et al, 1978., Dyerberg & Sinclair, 1980). A breadth of experimental and epidemiological studies have been carried out since then, to further investigate *n*-3 PUFA's, and their effect on cardiovascular health and disease. Many of these studies suggest there is an inverse relationship between increased *n*-3 PUFA intake and the incidence of mortality and morbidity (Rissanen et al, 2000). The cardio protective effects of fish oil are related to a number of mechanisms including reduced inflammation, enhanced endothelial function (Shah et al, 2007), decreased blood pressure (Tousoulis et al, 2014), reduced heart rate (Mozaffarian et al., 2005), and enhanced myocardial oxygen efficiency (Pepe, 2002).

The therapeutic action of *n*-3 PUFA on cardiovascular factors and inflammation in the health setting brings into question whether some of these actions are transferable to healthy individuals. It has been recognised that *n*-3 PUFA's may confer benefit in the athletic context (Simopoulos, 2007). A number of studies have shown that dietary fish oil supplementation may improve a variety of cardiovascular factors that could possibly influence exercise performance. Such factors include the enhanced deformability of erythrocytes (RBC deformability), increased artery diameter, and improved blood flow during exercise (Walser et al, 2008). Such modifications may lend themselves to improved oxygen delivery and potentially enhanced performance. Recent studies in young healthy participants confirm that increased dietary *n*-3 PUFA can modulate a number of cardiovascular mechanisms. *N*-3 PUFA supplementation has lowered HR at submaximal and peak exercise intensities and reduced whole body oxygen consumption during submaximal exercise (Peoples et al , 2008). Further observations include reduced rating of perceived exertion (RPE), reduced diastolic blood pressure and heart rate at submaximal exercise intensities (Buckley, Burgess, Murphy, & Howe, 2009). Many studies have reported positive effects of *n*-3 PUFA supplementation on cardiovascular variables; however, it has not yet been substantiated if these effects translate into performance improvements. *N*-3 PUFA supplementation for 3 weeks (6g/ day) did not improve $\dot{V}O_{2\max}$, max power or a performance trial (time to complete a set amount of work) in trained healthy cyclists compared to the placebo group (Oostenbrug et al, 1997). *N*-3 PUFA supplementation failed to influence the performance of a treadmill run to exhaustion in Australian rules footballers (Buckley et al, 2009) or the performance of a cycling time trial in young healthy adults (Da Boit et al, 2015). However, Zebrowska et al. (2015) reported that 1.1g/ day *n*-3 PUFA significantly increased $\dot{V}O_{2\max}$ and endothelial function in elite male cyclists. Further

work is warranted to investigate *n*-3 PUFA and cardiovascular benefits in the exercising population.

N-3 PUFAs exert an immunomodulatory effect reducing the production of inflammatory mediators, and are responsible for the production of anti-inflammatory mediators known as protectins, resolvins and maresins (Chapkin, et al, 2009). *N*-3 PUFA may act as a nutritional countermeasure to exercise induced inflammation (Simopoulos, 2007). Inflammation is the normal biological response to injury, infection and stress, and in the athletic context, it is necessary for adaptation to exercise. However when inflammation becomes excessive and uncontrolled it can contribute to tissue damage disease and the loss of physiological function. (James, Gibson, & Cleland, 2000). In previous studies, much attention has been focused on circulatory cytokine concentrations to examine the extent of the exercise induced pro and anti-inflammatory response. Much of the research has investigated long duration endurance exercise such as marathon and ultra-marathon events (Nieman et al., 2003), down-hill running and eccentric exercise (Peake, 2005). After a marathon race, inflammatory cytokines, IL1 β and TNF α increased 1.5 and 2 fold respectively and IL-6 increased markedly (100 fold) (Ostrowski, 1998). These events produce extensive muscle damage likely to cause large changes in the cytokine/ immune response. Elevated TNF α and IL1- β levels have been reported following 1.5 and 2 hours of cycling (Cox et al., 2010; Brenner et al., 1999 respectively). However, in several studies, changes in systemic levels of inflammatory TNF α and IL1 β could not be detected or only very small changes were measurable in plasma after eccentric exercise (Smith, 2000; Hirose, 2004). Ostapiuk-Karolczuk et al. (2012), observed increases in IL-6 and anti-inflammatory IL-4 immediately post 90 minutes running at 65% $\dot{V}O_{2\max}$. There are a number of explanations for the equivocal results regarding pro and anti-inflammatory responses to exercise. These are related to the mode/ intensity and duration of

exercise as well as the sensitivity of the assay used to analyse cytokine concentrations (Pedersen & Hoffman Goetz, 2000). Although the majority of previous research has examined the inflammatory response to heavy prolonged or eccentric exercise, it remains to be elucidated if short term cycling exercise results in an acute inflammatory or anti-inflammatory cytokine response. Therefore, the first purpose of this thesis was to investigate the acute cytokine response to cycling sessions of differing duration and intensity. More specifically to investigate the cytokine response to a 1-hour preloaded time trial and a high intensity interval session. It was hypothesized that both trials would produce an increase in IL-6 and inflammatory cytokines, and the increase would be more marked in the 1 hour cycling trial.

The second purpose of the thesis was to develop a suitable dietary intervention to increase erythrocyte phospholipid incorporation of *n*-3 PUFA. Previous researchers in the exercise field have employed a range of supplementary protocols ranging from 1.3g of *n*-3 PUFA per day (660mg EPA and 440mg DHA) for 3 weeks (Zebrowska et al., 2004), to 6.5g of *n*-3 PUFA per day (2000mg EPA and 1300mg DHA) (Raastad et al., 1997). *N*-6 PUFA and *n*-3 PUFA compete for enzymatic conversion and incorporation into tissues, therefore, lowering background dietary intake of *n*-6 PUFA could augment the incorporation and action of *n*-3 PUFA. No studies have evaluated the effect of lowering the background dietary intake of *n*-6 PUFA whilst simultaneously increasing *n*-3 PUFA in the exercise setting. It was hypothesised that an 8-week integrated dietary intervention to increase *n*-3 PUFA intake and to reduce the background dietary *n*-6 PUFA intake (H3-L6) would increase erythrocyte incorporation of *n*-3 PUFAs, EPA and DHA and would reduce *n*-6 PUFA, AA and LA.

The third purpose of this thesis was to investigate the effect of (H3-L6) on oxygen consumption during submaximal exercise in trained men. Previous researchers

investigating *n*-3 PUFA and its effect on oxygen consumption have based their findings on $\dot{V}O_2$ or $\dot{V}O_{2\max}$ measurements (Raastad et al., 1997; Zebrowska et al., 2004; Peoples et al., 2008; Da Boit et al., 2015). Whole body O_2 consumption measured via expired gases closely reflects O_2 consumption in the active muscles during steady state light to moderate exercise (Grassi 1996). However, inferences on the relationship between $\dot{V}O_2$ and $M\dot{V}O_2$ can be made using near-infrared spectroscopy (NIRS), therefore muscle oxygen delivery and extraction will be examined in addition to $\dot{V}O_2$. It was hypothesised that *n*-3 PUFA supplementation would reduce oxygen consumption during submaximal cycling and would increase $\dot{V}O_{2\text{ peak}}$.

The following review of literature will discuss some of the available research in relation to *n*-3 PUFA and its action in the clinical and exercise setting.

CHAPTER 2 REVIEW OF LITERATURE

2.1 SCOPE OF THE REVIEW

The potential beneficial effects of *n*-3 PUFA on the maintenance of health and disease states has become an increasingly popular and important area of research. In the past 50 years, epidemiological and clinical studies have suggested that increasing dietary intake of *n*-3 PUFA (specifically eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, C22:6 *n*-3) is associated with a number of improvements in health status. Previous studies have focused their attention on *n*-3 PUFA and their role in cardio protection, antiarrhythmic effects, lowering of plasma, triglycerides (GISSI – Prevenzione investigators, no author, 1999, cited in Rissanen, et al, 2000), anti-thrombotic, and reduced atherosclerotic effects (Calder, 2012). In addition, studies have examined *n*-3 PUFA and its effects on endothelial relaxation, lowering of blood pressure and its anti-inflammatory effects (Mozaffarian & Wu, 2011). The foundation of these potential *n*-3 related mechanisms are embedded in clinical, health related studies. Few human studies have examined the inflammatory response to endurance exercise, particularly in relation to cycling. In addition, few studies have examined *n*-3 PUFAs and their effect on exercise related variables, performance or capacity. The following literature review will introduce the nature of *n*-3/ *n*-6 PUFAs, dietary sources and metabolism. The theoretical basis of *n*-3 PUFA and its modulation of inflammation, and cardiovascular mechanisms will be discussed, and a review of relevant *n*-3 and exercise related studies will be presented.

2.2 OMEGA-3 AND 6 FATTY ACIDS, DIETARY SOURCES & METABOLISM

Omega 3 and 6 fatty acids are essential fatty acids (EFA) in the diet. The human body cannot synthesize them without regular consumption. The essential omega 6 and omega 3 fatty acids are linoleic acid (LA) (18:2 *n*-6) and alpha linolenic acid (ALA) (18:3 *n*-3) respectively. It is possible to distinguish a polyunsaturated fatty acid (PUFA) as *n*-3 or *n*-6 PUFA by the position of the first double bond in its carbon chain (see figure 2.1). LA is found in dietary sources such as plants and seeds, ALA is mainly found in green leafy vegetables (Aarsetoey et al., 2012), in oils such as soybean oil, rapeseed oil and some nuts and seeds including flaxseeds and walnuts (Graham & Burdge, 2005). EFA's are metabolised or elongated into longer carbon chain PUFA's of 20 and 22 carbons by the addition of double bonds. In humans, arachidonic acid (AA *n*-6) is metabolised from LA.

Figure 2.1. Structural formulas for *n*-3 PUFA α linolenic acid and *n*-6 PUFA linolenic acid Simopoulos (1991). FIGURE REDACTED

2.2.1 OMEGA-3 AND 6 ELONGATION AND SATURATION

Eicosapentaenoic acid (EPA; 20:5 *n*-3) and docosahexaenoic acid (DHA; 22:6 *n*-3) are two important fatty acids related to human health. Both EPA and DHA can be synthesized from ALA by desaturation and elongation in the liver (Mozaffarian & Wu, 2011) (see figure 2.2). Since both *n*-3 PUFA and *n*-6 PUFA metabolism occurs using the same pathway, they potentially compete for the same desaturation and elongation enzymes. However, desaturase enzymes 4 and 6 preferentially elongate *n*-3 PUFA when sufficient substrate is available in comparison to *n*-6 PUFA (Mickleborough, 2008). ALA conversion to EPA and DHA in humans is estimated to

be approximately 8 – 20% (EPA) and 0.5 – 9% (DHA). EPA and DHA concentrations in tissue cells and plasma are much higher than ALA (Graham & Burdge, 2005). Typical consumption of ALA (*n*-3) is approximately 10-fold lower than LA (*n*-6). Table 2.1 demonstrates how intake can vary greatly across different sample populations.

Figure 2.2 Essential fatty acid metabolism desaturation and elongation of *n*-6 PUFA and *n*-3 PUFA. Simopolous (1991). FIGURE REDACTED

Table 2.1 Adult consumption of LA and ALA in a selection of countries Graham & Burge, (2005). TABLE REDACTED

2.2.2 *N-3 PUFA'S AND CURRENT RECOMMENDED INTAKES*

Current international health guidelines suggest a number of different recommendations for *n-3* PUFA intake. Some are based upon volume of fish consumed, for example, the American Heart Association guidelines (see table 2.2) and others are based on approximate milligrams or grams per day (Scientific Advisory Committee on Nutrition, United Kingdom see table 2.2). The literature regarding specific dietary recommendations for the intake of *n-3* PUFAs, EPA/ DHA, and other *n-3* PUFAs is equivocal. There is little consensus regarding the optimal daily intake for a number of reasons. Studies carried out on *n-3* PUFAs have focused on many different types of healthy and disease state populations and the mechanisms assessed in these studies vary. It is known that EPA and DHA incorporate into different tissues at different rates. For example, EPA is incorporated more efficiently into erythrocyte membranes than DHA (Gans et al., 1990). In skeletal muscle phospholipids, a significantly higher amount of *n-3* PUFA incorporation has been observed in trained versus untrained athletes, independent of dietary fatty acid profile or muscle fibre type. This could be explained by changes in fatty acid metabolism related to exercise training (Andersson et al., 2000., Helge et al., 2001). Therefore, optimal daily-recommended dosage would need to take into consideration the population of focus and the desired mechanism of effect. With reference to common *n-3* PUFA dosages adopted in the exercise literature, the higher end of daily intakes considered safe by the European Safety Authority (2-6g/ day) are often employed in an attempt to enhance performance or modulate cardiovascular variables (Lindley, 2007; Weylandt et al., 2015; European Food Safety Authority (EFSA), scientific opinion related to the tolerable upper intake level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) 2012). Studies that have provided daily doses in this range have observed enhanced brachial artery diameter and blood flow (Walser et

al., 2006; 2008) and reduced heart rate and oxygen consumption during submaximal exercise (Peoples et al., 2008). Studies have also reported no effect of *n*-3 PUFA supplementation on exercise performance, heart rate or oxygen consumption (Da Boit et al., 2015). Therefore, the mechanisms of *n*-3 PUFA and the dosage required to reveal them is equivocal.

Table 2.2 Recommendations for *n*-3 PUFA intake for the prevention of cardiovascular disease (Aarsetoey et al., 2012) TABLE REDACTED

2.2.3 EVOLUTION AND THE BALANCE OF OMEGA-6 AND OMEGA-3 FATTY ACIDS IN THE HUMAN DIET

Anthropological and epidemiological studies suggest that human dietary fat intake has changed significantly in the past 160 years. It is estimated that there is a deficiency of *n*-3 PUFA intake in the modern western diet with an unbalanced ratio of *n*-6 PUFA to *n*-3 PUFA of 15/ 1 to 16/ 1, rather than 1/1. This is the ratio observed in the diet of wild animals and is the presumed traditional ratio of fats required in the human diet (Simopoulos, 1991). Human metabolism requires both *n*-3 and *n*-6 PUFAs. EPA and DHA concentrations in plasma, cells and tissues can be increased with the consumption of oily fish for example sardines and mackerel, or through supplementation with fish oil/ krill oil (Graham & Burdge, 2005., Tou et al., 2007). Increased *n*-3 PUFA intake results in changes in tissue fatty acid composition. In particular, the competitive incorporation of *n*-3 PUFA in relation *n*-6 PUFA in cell phospholipids (Mickleborough et al., 2008). This influences the physical properties of the cell resulting in modification in the activity of cell signalling, membrane receptors, gene expression and the production of lipid mediators (Calder, 2006). Table 2.3 details a more comprehensive list of some of the biological mechanisms associated with EPA and DHA.

Table 2.3 some of the proposed mechanisms and biochemical effects of *n*-3 Polyunsaturated fatty acids (*n*-3 PUFAs) (Aarsetoey et al., 2012) TABLE REDACTED

2.3 THEORETICAL BASIS FOR *N*-3 PUFA SUPPLEMENTATION AND ITS EFFECT ON INFLAMMATION

Inflammation is the normal biological response to injury, infection and stress and is necessary for adaptation to exercise. Although inflammation is a normal response, when it becomes excessive and uncontrolled it can reduce physiological function. (James et al., 2000). Increased levels of inflammatory mediators such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, and IL-6 are destructive, chronic overproduction can cause issues related to muscle wasting and loss of bone mass (Calder, 2006). Limited recovery during times of increased physiological stress in athletes may lead to local inflammation developing into chronic systemic inflammation characterised by chronic fatigue and or the prevention of exercise adaptation (Gleeson, 2006). This can be further provoked when the diet is high in *n*-6 relative to *n*-3 PUFA, when arachidonic acid (AA), becomes the primary lipid incorporated into immune cell membrane phospholipids. The AA derived inflammatory pathway involves the production of AA derived eicosanoids, prostaglandins, leukotrienes and other factors related to inflammation. AA is metabolised by cyclooxygenases (COX), an enzyme responsible for the production of biological mediators known as prostanoids (Lindley, 2007). From AA, monocytes synthesise prostaglandin E_2 (PGE_2) and 4 series leukotrienes. When the diet is enriched with *n*-3 PUFA EPA and DHA for a period of weeks to months, these long chain PUFA's are preferentially incorporated in to cell membranes and reduce the production of AA derived PGE_2 and 4 series leukotrienes (Lee et al., 1985). This is due competitive inhibition of the metabolism of AA when dietary EPA and DHA increases (Calder, 2007) (see figure 2.3).

Figure 2.3. The commonly proposed anti-inflammatory effect of *n*-3 PUFA's via competitive inhibition of the AA cascade and Cox 2 pathway. (Cleland et al. 2005).

FIGURE REDACTED

A greater abundance of *n*-3 PUFA EPA and DHA in immune cells increases the production of EPA derived inflammatory mediators, and eicosanoids such as PGE3 and 5-series leukotrienes. The significance of this particular cascade is that EPA derived inflammatory mediators are less inflammatory in comparison to those derived from AA (Bagga et al., 2003). Therefore, changes in immune cell phospholipid composition may affect the function of these cells including changes to cell signalling pathways, and the type of inflammatory and anti-inflammatory mediators produced (Calder, 2007). In addition, EPA and DHA are precursors of a group of mediators, resolvins, protectins and maresins, which play a role in resolving the inflammatory response (Calder 2012). EPA and DHA produce the series E and D resolvins by aspirin-acetylated COX-2 produced in the endothelial cells of the vasculature, and by 5-LOX (in leukocytes). DHA also forms protectins in leukocytes. These mediators may decrease the production of inflammatory cytokines (Weylandt, 2012). Cytokines are a category of proteins known to be important as signals for cellular communication and in mediating immune function (Peake et al,

2005). They are produced locally in response to tissue damage, injury and infection. These cytokines, (some of which can be pro inflammatory and anti-inflammatory) coordinate the arrival of immune cells to treat the inflammatory response including monocytes, lymphocytes and neutrophils (Pederson, 2000). In exercise studies, the cytokines most commonly measured include, Interleukin – 1 (IL-1), Interleukin - 6 (IL-6) and Tumour Necrosis Factor alpha (TNF- α). Prostaglandin E₂ is also an important mediator of the production of inflammatory cytokines (Ostrowski et al, 1998).

2.4 STUDIES THAT HAVE EXAMINED THE INFLAMMATORY CYTOKINE RESPONSE TO EXERCISE

One of the main mechanisms by which *n-3 PUFA* may influence exercise performance and recovery is through modulation of inflammation. It is therefore important to consider the inflammatory cytokine response to exercise in particular regarding endurance cycling. The latter part of this literature review focuses on the effects of increased dietary *n-3 PUFA* on oxygen consumption in trained cyclists. It is important for us to understand the cytokine response to exercise, as it may be an underlying factor influencing the cardiovascular response to fish oil supplementation. The following review examines the typical cytokine response to a variety of different exercise modalities.

Since the 1980s a breadth of studies have examined immune function and inflammatory responses to high intensity interval training (Croft et al., 2009; Robson Ansley, 2006), endurance exercise (Suzuki et al., 2000; Nieman et al., 2001; Ostrowski, 1999) and intermittent team sport based exercise (Elliakim et al., 2009). Inflammatory responses to over training, overreaching and underperformance syndrome (UPS) have also been of interest (Halson et al, 2003). Evans et al. (1986)

examined the effects of eccentric cycling for 45minutes duration on plasma IL-1 levels. 3 hours post exercise, IL-1 levels increased in all five untrained men with no change in trained men suggesting that training may exert a degree of adaptation to lowering the inflammatory response. In early studies, IL-1 was identified as the primary inflammatory cytokine responsible for post exercise induced plasma changes identified by researchers such as Cannon and Kluger (1983) and Evans et al (1986). However, these studies were carried out using a thymocyte proliferation bioassay (prior to the availability of recombinant IL-1 proteins) which also measures IL-6; therefore, IL-6 may have been responsible for the induction of inflammation. In follow up to this, there are a number of studies that reported no significant exercise induced increase in IL-1 levels in plasma (Cannon et al, 1991; Halkjaer & Pederson 1994).

The majority of studies dated pre 2000 that examined the inflammatory response to exercise considered one mode of exercise with a single group of participants. Brenner et al. (1999), was the first study that examined the cytokine response to three different types of exercise in the same group of participants. Eight moderately fit males were randomly allocated to four exercise conditions – All out (5 minutes of cycle ergometer exercise at 90% $\dot{V}O_{2\max}$), circuit training (Standard Circuit training routine including 5 types of exercise, 3 sets of 10 at 60 -70% RM), long (two hours of cycle ergometer exercise at 60 - 65% $\dot{V}O_{2\max}$), control (participants remained seated for five hours). Blood samples were analysed for creatine kinase (CK), natural killer cell counts (NK) and plasma levels of Interleukin-6 (IL-6), Interleukin 10 (IL-10) and tissue necrosis factor (TNF α). CK levels were elevated 72 hours following circuit training. NK counts increased significantly during all three types of exercise, the largest increase was in relation to all out exercise and the lowest increase was observed in the long exercise bout. NK values returned to pre exercise baseline levels within 3 hours of recovery. In Brenner et al. (1999), cytokine

responses were most marked in relation to the long exercise bout, which resulted in a significant increase in plasma IL-6 (peaking at 3 hours post exercise) and TNF α (peaking at 72 hours into the recovery period). No changes were observed in response to circuit training exercise. IL-6 is known to increase in response to prolonged exercise in an exponential fashion (Gokhale, 2007). Prolonged physical exercise or exercise that induces muscle damage is associated with elevations in pro-inflammatory cytokines such as IL-1 and TNF α (Moldoveanu et al., 2000). Increases in TNF α post exercise have been reported in response to prolonged exercise such as marathon running (Camus et al., 1997), 250-km road cycling (Gannon et al., 1997) and following 2 hours of cycle ergometer exercise at 60% $\dot{V}O_{2\max}$ (Brenner, 1999). In contrast, TNF α and IL1 β could not be detected, or only very small changes were measurable in plasma after eccentric exercise (Smith, 2000; Hirose, 2004). A possible explanation for variable findings in previous research may be due to differences in mode and duration of exercise. Hirose (2004) employed 6 sets of 5 eccentric actions of the elbow flexors at 40% of maximum isometric strength, while Smith (2000) employed 4 sets of 12 eccentric bench press and leg curl repetitions at 100% 1 repetition max (1RPM). Although these protocols are damaging they are not as prolonged as marathon running, distance cycling, and therefore may not have been of sufficient stimulus to evoke an inflammatory response. Peake (2005) investigated plasma cytokine changes in relation to exercise intensity and muscle damage in well-trained male runners and triathletes. The exercise conditions included 1) treadmill running for 1 hour at 60% $\dot{V}O_{2\max}$, 2) treadmill running for 1 hour at 80% $\dot{V}O_{2\max}$, and 3) downhill treadmill running at 10% gradient for 45 minutes at 60% $\dot{V}O_{2\max}$. Blood samples collected immediately pre and post exercise indicated that treadmill running at 80% $\dot{V}O_{2\max}$ had a greater effect than 60% or downhill running on increasing plasma concentrations of IL-1ra,

IL10, IL-12, and PGE₂. Both Brenner (1999) and Peake (2005) appear to indicate that inflammation is most likely to occur following long and intense exercise rather than exercise of shorter more moderate intensity and duration.

The inflammatory response may also be modulated by the health state of an individual. For example, Cox (2007), examined the cytokine response to three treadmill-running tests in healthy and illness prone runners. On three separate occasions, participants were required to run for 30 and 60 minutes at 65% $\dot{V}O_{2\max}$, and to perform 6 x 3 minute intervals at 90% $\dot{V}O_{2\max}$. Post exercise anti-inflammatory cytokines IL-10 and IL1-ra concentrations were lower in the illness prone group and the IL-6 response was 84 – 185% higher.

More recently, Landers-Ramos (2014) investigated the cytokine response to aerobic exercise in trained and untrained men. 10 endurance trained and 10 healthy males completed a 30-minute treadmill run at 75% $\dot{V}O_{2\max}$. A comparison of pre to post exercise cytokine values indicated a 165% increase in IL-6 post compared to pre exercise however there was no significant difference between groups in values for TNF α , IL-6, VEGF, bFGF, PIGF, or sFIT-1.

It would appear from the literature reviewed thus far, that prolonged exercise induces several components of the inflammatory response with significant increases in TNF α , IL-6. (Nieman, 2000; Calle & Fernandez, 2010). Differences in findings across studies are largely due to a number of factors for example the exercise mode/type of muscle contraction and intensity of effort across trials. The training status and age of participants (Gokhale, 2006) and the health of the participants (for example the number of episodes of upper respiratory symptoms per year) (Cox, 2007). In addition, the timing of blood sample collection, post exercise varies in previous work.

When considering the sequence of appearance of anti-inflammatory and pro-inflammatory cytokines it has previously been established that IL-6 is produced and appears in the circulation following the onset of exercise. This is followed by the production of pro-inflammatory TNF α and IL-1 β , and then the anti-inflammatory cytokines such as IL-4 and IL-10 (Koch, 2007; Peak et al., 2005). Ostapiuk-Karolczuk et al. (2012), aimed to clarify the time sequence of cytokines and inflammatory mediators following the performance of 90-minute running trial at 65% $\dot{V}O_{2\text{ max}}$ in healthy participants. IL-4, IL-6 and IL-10 increased significantly following exercise. Pro inflammatory cytokines IL-1 β and TNF α were detected 6 hours post exercise. It was concluded that exercise stimulated the production of inflammatory cytokines first (within approximately 20 minutes post exercise), and anti-inflammatory cytokines were produced in the hours following exercise. In previous studies, cytokines were measured at regular intervals such as 6, 12, 24 and 72 hours post exercise or immediately pre and post exercise. In addition, participant fatigue levels and feeding instructions prior to testing can affect hormone levels and subsequent cytokine elevation. For example when carbohydrate is ingested prior to or fed during exercise the rise in IL-6 is attenuated (Nieman et al, 1998; Steesberg et al, 2000). Steesberg et al. (2001), reported that IL-6 concentration was elevated earlier in a glycogen depleted leg (60 min following the onset of exercise), compared to a non-glycogen depleted leg (120 minutes following the onset of exercise). Therefore, muscle glycogen status is considered a stimulus for the transcription of the IL-6 gene.

The cytokine response to exercise and the specific mechanisms involved are not clear. Further research is required in both high intensity and endurance exercise to establish a model of the typical inflammatory response for trained athletes. In addition, there is no controlled study to date (of our knowledge) that has looked at the inflammatory response specifically to cycling exercise at different intensities.

The majority of previous studies have addressed cytokine responses to exercise known to induce an extreme inflammatory response and muscle damage such as marathon running (Camus et al, 1997) 250-km road cycling (Gannon et al 1997), treadmill running at high and low intensities of differing durations & downhill running (Pederson 2005). The intention of the initial study in this thesis was to investigate cytokine responses to both interval and 1 hour cycling exercise. This would provide important information regarding the extent of cytokine changes that occur in response to different cycling intensities and durations.

2.4.1 THEORETICAL BASIS FOR N-3 PUFA SUPPLEMENTATION AND ITS EFFECT ON EXERCISE PERFORMANCE AND RELATED PHYSIOLOGICAL VARIABLES

It has been established that *n*-3 PUFAs appear to have anti-inflammatory properties due competitive inhibition of the metabolism of AA when dietary EPA and DHA increases (Calder, 2007). In the exercise field there is also interest in the cardiovascular effects of *n*-3 PUFA supplementation and it's potential to lower exercising heart rate and whole body oxygen consumption (Peoples et al., 2008; Hingley., 2017). Furthermore, there is evidence to suggest that *n*-3 PUFA may improve endothelial function possibly due to increased nitric oxide production (Zebrowska, 2014). Together these findings suggest that *n*-3 PUFA may modulate some of the cardiovascular factors know to influence endurance performance (see fig 2.4). Endurance trained athletes employ a variety of training techniques to enhance exercise performance. Successful endurance performance (in this instance, endurance cycling) is dependent on the athlete's ability to maintain power or velocity over the duration of performance. Elite road cycling time trial races can vary from 10 minutes to approximately 60 minutes (for a 50 km time trial). Based on typical duration, time trials are predominantly dependent on aerobic metabolism

(Støren et al., 2013) which consists of the components, $\dot{V}O_{2\max}$ and exercise economy (Joyner & Coyle 2008). During training and racing, athletes are often working at high fractions of oxygen consumption (performance $\dot{V}O_2$) and maximal oxygen consumption ($\dot{V}O_{2\max}$) (Joyner & Coyle, 2008). It has been reported that professional road cyclists can perform workloads of approximately 90% $\dot{V}O_{2\max}$ for time periods of 60 minutes or more (Lucia et al., 2002). An athlete's $\dot{V}O_{2\max}$ (which for elite athletes is in the range of 70 – 85 ml kg⁻¹ min⁻¹) is a product of the ability of the heart to maintain a high cardiac output and increase stroke volume (Martin et al, 1986). It is also determined by the oxygen carrying capacity of the blood, the volume of blood flow to the muscle and the extraction of oxygen from the blood by the exercising muscles (Saltin & Strange, 1992; Bassett and Howley, 2000). The oxidative capacity of the muscle is also important regarding an individual's lactate threshold, and where this occurs in relation to their $\dot{V}O_{2\max}$ (Joyner and Coyle, 2008) (see figure 2.3). *n*-3 PUFA supplementation may assist in modulating some of the physiological factors that relate to endurance performance particularly submaximal oxygen uptake, maximal oxygen uptake and performance $\dot{V}O_2$. This is based on previous evidence that *n*-3 PUFA supplementation increased artery diameter and improved blood flow during exercise (Walser et al, 2006), lowered HR at submaximal and peak exercise intensities and reduced whole body oxygen consumption during submaximal exercise (Peoples et al , 2008). In addition *n*-3 PUFA supplementation has improved oxygen economy, reduced rating of perceived exertion (RPE), reduced diastolic blood pressure and heart rate at submaximal exercise intensities (Buckley et al., 2009).

Fig 2.4. Schematic diagram of the multiple physiological factors that interact as determinants of performance, velocity or power output. Joyner & Coyle (2008).

FIGURE REDACTED

Previous research regarding *n*-3 PUFA supplementation and its effects on endurance performance have commonly employed a test of oxygen efficiency, whole body oxygen consumption and $\dot{V}O_{2\text{ max}}$ tests in their methods (Oostenbrug et al., 1997; Raastad et al., 1997; Zebrowska, 2004). In the following section, we will review some of the main *n*-3 PUFA mechanisms that relate to the endurance performance model.

2.4.2. EPA AND DHA EFFECT ON THE VASCULAR FUNCTION AND NO

The vascular endothelium is an important contributor to circulatory homeostasis. Since the 1970s, the dynamic properties of the vasculature have become better understood. *N*-3 PUFAs have a therapeutic effect on endothelial dependent vascular function (Tagawa et al., 2002) including improved vasoregulatory

properties of the endothelium (Mozaffarian & Wu, 2011). In addition, *n*-3 PUFAs are theorised to reduce oxidative stress and damage to endothelial cells (Tagawa et al, 2002). In clinical research, it is known that patients with atherosclerosis or coronary artery disease have impaired brachial artery endothelial dilation. Ultrasound has been employed in studies to examine flow mediated dilation and the dilation of coronary vessels by acetylcholine (Neunteufl et al., 2000). DHA and EPA supplementation has been shown to enhance acetylcholine - induced vasodilation in forearm and coronary vessels, suggesting there is a therapeutic benefit in disease states (Fleischhauer et al 1993). Improvements in endothelial function accompanied with a decrease in resting heart rate have been observed in healthy volunteers following supplementation with 300mg of EPA and 200mg DHA per day for two weeks. This effect was also accompanied by a reduction in resting heart rate without affecting mean arterial pressure. It was concluded that it was likely that *n*-3 PUFA fatty acids may have changed the fluidity of endothelial cell membranes, promoting enhanced production or release of NO (Shah et al., 2007). *N*-3 PUFA is also theorised to augment skeletal muscle blood flow by the blunting of norepinephrine, which influences vasoconstriction in the muscle (Chin et al, 1993). The mechanism responsible for improved endothelial dependent vasodilation (EDV) is elusive. Vascular studies have demonstrated that fish oil can improve endothelial function and vascular tone in healthy individuals (Khan et al., 2002). It is possible that effects may be due to increased production and or release of endothelium derived NO (Black, 1979), increased expression of nitric oxide synthase (Nishimura et al, 2000), improved sensitivity of vascular smooth muscle cells to NO, and lowered production of endothelial adhesion molecules reducing leukocyte-endothelial interaction (Robinson & Stone, 2006). The latter may be a result of the anti-inflammatory action of *n*-3 PUFAs. EPA competes with the cyclooxygenase pathway producing a less inflammatory series of prostaglandins, thromboxanes, prostacyclins and

leukotrienes for example decreased IL-6, and TNF α (Mickleborough, 2013; Wu and Meininger, 2002). *N*-3 PUFA supplementation reduces platelet aggregation inflammation and vasoconstriction (Simopoulos, 2007). This raises questions regarding the potential of *n*-3 PUFA supplementation to facilitate blood flow in the exercising individual and to reduce the production of exercise induced inflammatory mediators. Attenuated vasoconstriction may also be the reason for the reported reduction in systemic vascular resistance and systolic/ diastolic blood pressure following *n*-3 PUFA supplementation (Mozzafarian et al, 2005).

Walser et al. (2006) reported that 6 weeks of dietary supplementation with DHA and EPA enhanced brachial artery dilation and blood flow during rhythmic handgrip exercise at 30% of MVC in healthy males and females. This provides some support for the augmented blood flow observed in previous health related studies (Shah et al, 2007) and the potential for this to occur in the exercise setting. It can be argued however that Walser et al, (2006) used an exercise mode that recruited a small muscle mass which makes it difficult to infer if similar results would be observed in endurance exercise where a larger muscle mass is recruited. Walser and Stebbins (2008), reported that a 6-week supplementation intervention with 3g EPA and 2g DHA per day enhanced stroke volume and cardiac output during 10 min moderate cycling exercise in healthy subjects. In addition, there was a trend for a decrease in systemic vascular resistance (SVR) in healthy subjects. *N*-3 PUFA may have attenuated vasoconstriction via the sympathetic nervous system and/ or augmented skeletal muscle vasodilation during exercise. These studies provide further evidence that *n*-3 PUFAs may potentially enhance blood flow and oxygen delivery during exercise.

It is also important to consider the effects of training on study outcomes. Training results in structural and functional remodelling of arteries “the athlete’s artery”. It has been suggested that training can influence endocrine and or neural factors that

control how arteries respond (Green, et al, 2012). Therefore, the results obtained from studies on healthy participants may not necessarily be transferable to the trained individual.

2.4.3. EPA AND DHA EFFECT ON CARDIAC MYOCYTES AND HEART RATE

The beneficial effects of *n*-3 PUFAs specifically EPA and DHA and their relation to cardiovascular health was discovered in the late 1960's when it was observed that the Inuit population who consumed a diet rich in *n*-3 PUFA, had low incidence of coronary heart disease and myocardial infarction (Mozaffarian & Wu, 2011). This study was followed up with large epidemiological trials such as the GISSI Prevenzione trial (No Author, 1999 cited in Ninio, et al, 2008) that provided further evidence to support the therapeutic effects of *n*-3 PUFAs on myocardial infarction patients. As a result of this, the American Heart association (AHA) produced a scientific statement recommending that individuals with coronary artery disease, or elevated triglyceride levels should consume >1g/ day of *n*-3 PUFA's (EPA + DHA) to reduce the risk of a coronary event and to maintain cardiac function (Kris-Etherton et al., 2003). Supplementation with EPA and DHA may modulate HR through a number of mechanisms. A breadth of health related studies has examined *n*-3 PUFA supplementation and its antiarrhythmic effect (Mozaffarian & Wu, 2011). Ex vivo cell studies have examined the stabilizing effects of *n*-3 PUFA on cardiac myocytes and in a meta-analysis of randomised controlled trials, *n*-3 PUFA supplementation reduced resting heart rate by 1.6 beats/ min (Mozzaffarian et al, 2005). Supplementation with *n*-3 PUFA increases cellular saturation of cardiomyocytes with EPA and DHA (Garg et al, 2006). This results in physical changes in the cardiac myocyte cell membrane by altering what is referred to as "rafts" and "caveolae" that hold membrane bound proteins, and fatty acids important for cell signalling (Endo & Arita, 2016). This modulates ion channel function for example Na⁺ and T-type

Calcium channels (Endo & Arita, 2016) and affects the generation of an action potential. *N*-3 PUFAs alter the kinetic properties of voltage gated sodium channels which results in an increase in the refractory period, decreasing the excitability of the myocardium (Leaf et al., 2003). This may be the reason why *n*-3 PUFA supplementation has been shown to increase left ventricular end diastolic volume and ejection fraction in primates, reducing rate pressure product and thus indicating improved oxygen efficiency of the myocardium ($\dot{M}\dot{V}O_2$) (McLennan et al., 1992, cited in Pepe & McLennan, 2002). Pepe et al. (2002) tested the effects of 16 weeks of *n*-3 PUFA supplementation on ventricular haemodynamics and $\dot{M}\dot{V}O_2$ in rodents. Isolated hearts were worked at a variety of loads. $\dot{M}\dot{V}O_2$ was reduced in the hearts of rodents (fed with an *n*-3 PUFA rich diet) whilst maintaining the same cardiac output and work done. This is indicative of improved ventricular function and myocardial oxygen efficiency. Interestingly this effect was observed at higher workloads and independent of changes in vascular function. This may indicate that *n*-3 PUFA directly improves the O_2 efficiency of the myocardium.

Several studies have reported that fish oil supplementation has reduced heart rate during submaximal exercise. This effect has been observed during, treadmill running to exhaustion (Buckley et al., 2009), 20 minutes cycling at low and moderate intensities (Walser et al., 2008), 1 hour of cycling at 55% $\dot{V}O_{2\text{ peak}}$ (Peoples et al., 2008) and 30 minutes cycling at intensities equivalent to 2 and 3-mM of blood Lactate (Kawabata et al., 2014). Conversely Da Boit et al., (2015) observed no changes in exercising heart rate. Da Boit employed a cycling protocol, where subjects performed a set workload at 70% $\dot{V}O_{2\text{ max}}$ (equating to approximately 90 minutes of exercise). It is unclear why these findings differed. Further research is warranted to investigate the effects of *n*-3 PUFA on factors such as HR and $\dot{V}O_2$

consumption during exercise at different intensities in the same population group.

Further detail on these studies is presented in section 2.5.

2.4.4. EPA AND DHA, SKELETAL MUSCLE AND MITOCHONDRIAL MEMBRANE COMPOSITION AND BIOENERGETICS

Dietary fatty acid intake is known to influence the incorporation of fatty acids into the body's stored and structural lipids for example, in blood serum, erythrocyte phospholipids, adipose tissue and skeletal muscle tissue (Andersson, 2002). *n*-3 PUFA incorporation into skeletal muscle tissue, may affect its metabolic function via anchoring of membrane proteins, and altering permeability and fluidity (Stubbs & Smith 1984). In addition, how much *n*-3 PUFA EPA and DHA is incorporated into cells may be dependent on the tissue type for example cardio-myocytes take up large amounts of DHA, in addition, DHA is preferentially incorporated into skeletal muscle membrane composition (Peoples & McLennan, 2010). Altering dietary PUFA intake is also thought to influence the DHA content and function of the lipid bilayer of whole skeletal muscle (Owen et al., 2004; Dangardt et al., 2012 cited in Herbst et al., 2014). DHA is known to be important in the cell membranes of mitochondria for the function and organization of proteins in the phospholipid bilayer (Tsalouhidou et al., 2006). Therefore *n*-3 PUFA may affect mitochondrial function and bioenergetics (Herbst et al., 2014). A limited number of studies have investigated how dietary *n*-3 PUFA supplementation influences mitochondrial membrane fatty acid composition and its function. There is evidence that *n*-3 PUFA incorporation into myocardial membranes reduces myocardium oxygen consumption ($\dot{M}\dot{V}O_2$) without any change in cardiac output or work done. These changes were due to improved coronary blood flow and oxygen extraction (Pepe et al, 2002). In addition, Peoples and McLennan (2008) found fish oil supplementation

lowered heart rate and reduced whole body oxygen consumption during submaximal exercise (55% of peak workload until fatigue). Changes in mitochondrial metabolism and improved calcium handling as a result of *n*-3 PUFA supplementation may have contributed to these findings (Pepe and McLennan 2002). Alternatively, there is evidence that *n*-3 PUFAs can shift substrate metabolism to preferentially utilise glucose, requiring less O₂ to break down per mole than fat (Frayn, 1983, cited in Peoples et al., 2008). The evidence to substantiate improved mitochondrial respiration following *n*-3 PUFA supplementation is elusive. Herbst, (2014) investigated the effects of *n*-3 PUFA supplementation on mitochondrial incorporation of fatty acids, mitochondrial bioenergetics and submaximal substrate metabolism in skeletal muscle (vastus lateralis). Measurement of O₂ consumption was performed on permeabilized fibres using an oxygraph-2K. ADP respiratory kinetics were determined in the presence of creatine. *N*-3 PUFA was readily incorporated into mitochondrial cell membranes but was not associated with maximal mitochondrial function. ADP sensitivity and ADP stimulated respiration (during submaximal exercise) was increased which could possibly improve the efficiency of ATP resynthesis during exercise, or may affect ATP synthase activity. The exact mechanisms remain ambiguous additional work needs to examine muscle *n*-3 PUFA's and mitochondrial bioenergetics.

2.4.5 EPA AND DHA AND EFFECT ON RED BLOOD CELL DEFORMABILITY

Red blood cell deformability refers to the resilience of RBC's to change shape under an applied force or sheer stress as they pass through the circulation. RBC deformability decreases during exercise, and this may negatively affect blood flow (Oostenbrug et al., 1997). Reduced RBC deformability is thought to be a result of free radical production in response to exercise, which in turn increases peroxidation of fatty acids (FA) in RBC phospholipids (Oostenbrug et al., 1997). Several studies

have found that supplementation with *n*-3 PUFA and its incorporation into RBC phospholipids reduces RBC deformability (Cartwright et al., 1985; Terano et al., 1983), enhancing the transport of RBCs through the circulation (Bruckner et al, 1987). Theoretically, *n*-3 PUFA supplementation may influence exercise performance or performance related variables by reducing the viscosity of the blood, in turn affecting peripheral blood flow and potentially cardiac output (Cartwright et al, 1985., Kobayashi et al, 1985). Both of these are important components that determine maximal aerobic capacity. It should be noted however, that well trained aerobic athletes generally have reduced blood viscosity compared to healthy subjects (Letcher, 1981). Previous research investigating the effect of *n*-3 PUFA on RBC deformability has been carried out on healthy subjects or clinical populations. Therefore, supplementation may affect athletes differently due to them already having lower viscosity levels at baseline. Guezennec et al, (1989) examined the effect of a 6 week supplementation period (delivering 1,080 mg EPA and 720 mg DHA/ day), on the characteristics of RBC membranes in 14 male subjects following hypobaric exercise (consisting of one bout of 1h cycling at 70% $\dot{V}O_{2\max}$ at sea level, and one bout at simulated altitude of 3000m in a hypobaric chamber). The exercise induced decrease in RBC deformability was suppressed in the *n*-3 PUFA supplemented athletes. In contrast (Oostenbrug et al., 1997), examined the effect of a 3-week *n*-3 PUFA supplementation period (6g/ day) on time trial cycling performance (1 hr time trial), RBC deformability and lipid peroxidation in trained cyclists. Exercise reduced RBC deformability, however fish oil supplementation did not alter this effect. In addition, there was no effect of *n*-3 PUFA on exercise performance. In long chain PUFA studies the effects of lipid peroxidation is an important consideration. RBC membrane *n*-3 PUFA content increases significantly following supplementation, therefore it is important to consider that the benefits of

n-3 PUFA could be counteracted by the negative effects of lipid peroxidation (Mickleborough, 2013).

2.5 OMEGA-3 FATTY ACIDS, HUMAN PERFORMANCE AND ASSOCIATED PHYSIOLOGICAL VARIABLES

A number of potential benefits of *n*-3 PUFA have been described, many of these effects have been observed in the health context and in disease states. Some of these factors, if translated effectively to the athletic population may have the potential to enhance physical performance. There is clear evidence that *n*-3 PUFA fatty acid supplementation may have the potential to modulate a number of endurance related physiological mechanisms such as, Improved myocardial efficiency, whole body oxygen efficiency, reduction in resting/ exercising heart rate and increased vascular dilation and blood flow. The following review of *n*-3 PUFA and endurance exercise literature is presented as a time line. *N*-3 PUFA and exercise induced inflammation is reviewed separately in section 2.6 as an underlying factor that may influence blood flow and cardiovascular mechanisms.

A limited number of human studies have been carried out that examine *n*-3 PUFA supplementation and exercise performance. Leaf and Rauch (1988) was the first study to report that fish oil supplementation of 6g per day increased treadmill based $\dot{V}O_{2\max}$ performance however; there was no observed benefit on $\dot{V}O_{2\max}$ when the daily dosage of fish oil was increased to 12g/day. An increase in $\dot{V}O_{2\max}$ following *n*-3 PUFA supplementation has not been confirmed by the majority of subsequent studies. Raastad et al. (1997) supplemented 15 male soccer players with 5.2g fish oil per day for 10 weeks. Plasma levels of EPA and DHA significantly increased however, no changes in $\dot{V}O_{2\max}$ performance were observed in the fish oil group compared to the placebo group. Leaf and Rauch (1997) originally used a percentage

grade treadmill test to examine $\dot{V}O_{2\max}$, with running maintained at a constant speed (modified Bruce protocol). Raastad (1997) criticized this protocol, as the participants running technique and motivation may have influenced $\dot{V}O_{2\max}$ values. This relevance of this point is further supported by an increase in maximal heart rate of 8 beats/ min observed in the low dose *n*-3 PUFA condition with no change at all observed when the fish oil dose was increased (Leaf and Rauch 1997). Due to the limitations identified, Raastad et al., (1997) adopted a step based protocol with treadmill speed increasing $1\text{km}\cdot\text{h}^{-1}$ at a constant grade of 3° . No differences were observed in exercise performance (maximal aerobic power, anaerobic threshold or running performance) or measures of red blood cell deformability in response to supplementation with *n*-3 PUFA. There was however a reduction in plasma triglyceride levels in the fish oil group. Zebrowska et al (2014) is the only other study that has reported an improvement in $VO_{2\text{peak}}$, accompanied by reduced heart rate at peak power and improved flow mediated dilation in cyclists supplementing with *n*-3 PUFA.

Oostenbrug et al., (1997) supplemented well-trained male cyclists with 3 weeks of fish oil 6g/ day or a placebo. The participants carried out a cycling time trial ~1 hr in duration (pre-fixed absolute workload based on 70% of W_{\max}). No differences were observed in W_{\max} , $\dot{V}O_{2\max}$, or time trial performance in the *n*-3 PUFA supplemented group. In addition, no change in blood cell deformability was observed.

Peoples et al. (2008) examined the effect of *n*-3 PUFA supplementation on O_2 efficiency in 16 well-trained male cyclists. No differences in peak O_2 consumption or peak workload was observed between groups pre or post supplementation. Heart rate was significantly reduced during increasing workload and at $\dot{V}O_{2\text{peak}}$ in the fish oil supplemented group. Whole body O_2 consumption and heart rate was significantly lower in the fish oil supplemented group compared to the control group

throughout a ~60min submaximal exercise test at sustained intensity (55% of peak) to exhaustion. In addition, rate pressure product (RPP) (heart rate x systolic blood pressure) was significantly lower during submaximal exercise in the fish oil supplemented group. This study indicated further evidence that *n*-3 PUFA modifies O₂ utilization by the heart and skeletal muscle. Improved mitochondrial efficiency may be one of the mechanisms contributing to improved efficiency. Peoples et al. (2008) observed no change in peak O₂ consumption in the *n*-3 PUFA supplemented group, this did not support the earlier findings of Leaf and Rauch (1997). Possible reasons for this may include the control Peoples et al. (2008) employed in their study by recruiting participants who were well trained and in a controlled phase of their training programme, this reduced possible fluctuations in peak O₂ consumption as a result of training adaptation. Peoples (2008) employed a submaximal ~60min (55% $\dot{V}O_{2\max}$) sustained exercise intensity test to exhaustion which is known to be affected by within subject variability and does not necessarily substantiate any performance improvement (Jeukendrup et al 1996). Time trials may be a better indicator of performance improvement however oxygen consumption is not the only limiting factor in the performance of higher intensity time trial protocols (Levine, 2008).

N-3 PUFA and its influence on blood flow has been considered as one of the contributing factors for improved exercise economy. Walser et al. (2006) previously demonstrated that *n*-3 PUFA EPA and DHA supplementation enhanced vasodilation and blood flow to the exercising skeletal muscle during repeated handgrip contractions. However, HR and BP responses were not examined. To effectively investigate the effects of *n*-3 PUFA supplementation on HR and BP, a larger muscle mass or exercise modality that uses the whole body is required. Therefore, Walser and Stebbins, (2008) examined the effect of 6 weeks supplementation of *n*-3 PUFA on HR, BP, SVR, SV and CO during a 20 min cycle ergometer test. The protocol

included 10 minutes of cycling at low intensity where parasympathetic tone was still present (HR 90 – 100 bpm), and 10 minutes at a moderate cycling intensity where vagal tone would be minimal and the sympathetic nervous system would be activated (HR \geq 125 bpm). *n*-3 PUFA supplementation increased SV and CO and tended to decrease SVR during moderate intensity cycling. A decrease in resting MAP pressure was observed, and there were no changes in heart rate during exercise or at rest. It has been suggested that fish oil reduces sympathetic norepinephrine-induced vasoconstriction in exercising skeletal muscle (Chin et al, 1993). This may also explain why these changes were observed in the moderate workload rather than the low intensity cycling load. Walser and Stebbins (2008), found resting mean arterial pressure (MAP) was reduced (88 ± 5 vs. 83 ± 4 mm Hg pre and post treatment respectively) this is a relatively small change and previous studies have also only recorded small changes of approximately $\sim 2\%$ (2-4mm Hg) (Geleijnse et al, 2002). It could be possible that the large increase in blood pressure that accompanies whole body exercise may mask any small *n*-3 PUFA influence. The short duration of the exercise protocol and the fixed intensity should also be taken into consideration, as it does not reflect the real life training and performance load of cyclists.

Few studies have examined the effect of *n*-3 PUFA supplementation directly on performance. Buckley et al. (2009) examined the effect of 5 weeks of *n*-3 PUFA DHA rich supplementation on endurance performance, recovery from exercise and HR in 29 professional Australian football league players. Erythrocyte *n*-3 PUFA content was almost doubled during the supplementation period however; it was not associated with any improvements in time trial performance or in recovery (measured by the performance of a subsequent time trial). Serum triglyceride levels and HR reduced during submaximal exercise by ~ 8 beats/ min however; there was no change in peak heart rate. This agrees with the findings of Peoples et al. (2008),

where a reduction in HR was observed during steady state exercise. However, Peoples et al. (2008) also observed reduced heart rate during incremental loads to exhaustion (including peak heart rate) in the *n*-3 PUFA supplemented group. Both Buckley (2009) and Peoples (2008), employed submaximal trials to exhaustion however different modes of exercise were performed, treadmill running and cycling respectively.

More recently (Nieman et al, 2009) employed a more multifaceted research design to examine the effect of 6 weeks of *n*-3 PUFA supplementation on exercise performance, inflammation and immune measures in trained cyclists, following 3 days of intense exercise. Each day consisted of steady state cycling for 3 hours (57% W_{max}) with a 10km time trial inserted in the final 15 minutes of each 3 hour session. There was no change observed in immune markers (blood leukocytes, C-reactive protein, creatine kinase, Salivary-IGA, and myeloperoxidase) or inflammatory mediators (IL-1ra, IL-6, and IL-8). No changes were observed in exercise performance. However, it should be noted that this study used a relatively low dose of fish oil 2.4g/ day (2g EPA and 0.4g/day DHA) in comparison to other studies (see table 2.4).

Zebrowska et al. (2014) evaluated the effect of 3-weeks of *n*-3 PUFA supplementation on $\dot{V}O_{2max}$ in 13 male elite cyclists. In addition, serum nitric oxide (NO), asymmetric dimethyl arginine (ADMA) concentration, and vascular indexes of brachial artery dilation were determined using ultrasound. The results demonstrated there was an improvement in $\dot{V}O_{2max}$ in the fish oil supplemented group and elevated resting and exercise serum NO levels in comparison to the placebo group. Furthermore elevated NO improved flow mediated dilation (FMD). It was concluded that incremental exercise stimulated NO production however; NO production was further increased following supplementation with *n*-3 PUFA. Improved vascular

dilation and blood flow was thought to influence the improvement in peak $\dot{V}O_2$ and peak $\dot{V}O_2 / HR_{max}$ changes observed in the study. In addition, *n*-3 PUFA reduced HR at maximal power in the *n*-3 PUFA supplemented group. These results are in agreement with Shah et al. (2007), where a 2-week intake of *n*-3 PUFA significantly increased brachial artery flow mediated vasodilation.

Krill oil is an alternative rich source of EPA and DHA supplementation. The DHA content of krill is similar to oily fish however; the EPA content is greater (Tou et al. 2007). Studies show higher *n*-3 PUFA incorporation into erythrocyte cell membranes with krill oil in comparison to fish oil supplementation (Ulven et al. 2011, cited in Salem & Kuratko, 2014). Da Boit et al. (2015), examined 6 weeks of krill oil supplementation on exercise performance and markers of immune function. 19 males and 18 females were randomly assigned to consume krill oil daily for 6 weeks (2g/ day) or placebo (2g/ day). No changes were observed in measures of immune function (IL-6, IL-10, IL-17 and $IFN\gamma$) post supplementation however, NK cell cytotoxic activity and mononuclear cell IL-2 production increased three hrs post exercise. In addition, as exercise intensity increased PGE-2 production also increased. The lack of an increase in other immune markers is in agreement with Yaqoob et al, (2000) and Gray et al, (2014). In addition, Da Boit, (2015) found no effect of Krill oil supplementation on time trial performance (time to complete a set workload), oxygen consumption or exercising heart rate. In previous human research studies, *n*-3 PUFA modification of heart rate or oxygen efficiency has typically been observed at lower exercise intensities. Da Boit et al, (2015) used a time trial workload that was equivalent to $\sim 70\% \dot{V}O_{2\max}$ (~ 10 min time trial). Due to ambiguous results in previous literature, it is clear that studies need to examine the effect of *n*-3 PUFA on HR and $\dot{V}O_2$ at a variety of different workloads in the same population. It should also be noted that the supplementation level of EPA and DHA

in Da Boit et al. (2015), was much lower than used in previous studies (see table 2.4). The authors also noted blood samples had not been taken during the baseline exercise test for direct comparison.

During submaximal steady state exercise oxygen consumption ($\dot{V}O_2$) is influenced by both cardiac output, an individual's oxygen carrying capacity and muscle oxygen uptake (Joyner & Coyle 2008). There is evidence that *n*-3 PUFA supplementation may improve cardiac and skeletal oxygen efficiency translating into reduced whole body oxygen use during exercise Raastad et al (1997). Peoples et al. (2008), previously reported DHA rich *n*-3 PUFA supplementation reduced the oxygen cost of submaximal intensity steady state exercise in trained male endurance athletes. Kawabata et al. (2014), questioned if it was the combined effect of aerobic training and fish oil consumption that resulted in these observations. Kawabata et al (2014) investigated the effect of EPA rich *n*-3 PUFA supplementation on exercise economy (EE), during moderate intensity submaximal exercise. The participants were non-aerobically trained men (in contrast to Peoples, (2008) who employed trained athletes). Half the group consumed fish oil supplements 3.6g FO per day containing 0.914g EPA and 0.399g DHA, while the placebo group consumed 3.6g of medium chain triglycerides. Pre and post supplementation participants completed a $\dot{V}O_{2\max}$ test and a steady state submaximal test on a cycle ergometer (30 minutes at 2 mmol lactate workload followed by 30 minutes at 3 mmol workload with a 10 min rest between sessions). EPA and DHA erythrocyte levels significantly increased in the *n*-3 PUFA supplemented group and a negative linear correlation was observed between the increase in erythrocyte EPA levels and whole body oxygen uptake during submaximal exercise. There was no significant change in $\dot{V}O_{2\max}$ pre to post supplementation. These findings regarding $\dot{V}O_{2\max}$ are in agreement with Raastad et al. (1997), and Peoples et al. (2008), who reported that 8g/ day of FO

supplementation did not affect maximal oxygen consumption. The observed improvement in oxygen efficiency during submaximal exercise in the *n*-3 PUFA supplemented group supports the improved exercise economy findings by Peoples, (2008). In addition, there were no significant changes noted in HR or VE. RPE was significantly reduced in the FO group in both the incremental exercise and during the steady state trial. FO may have contributed to participants performing the exercise at a lower perceived intensity. The observed improvement in exercise economy should be treated with some caution as random allocation of participants resulted in fitness differences between groups it was noted that the $\dot{V}O_{2\max}$ of the *n*-3 PUFA supplementation group (pre supplementation) was significantly lower than that of the control group, which may have affected their response to supplementation.

More recently, the research has moved towards the examination of *n*-3 PUFA mechanisms in conditions of increased exercise stress. Oxygen efficiency/ utilisation related mechanisms linked to *n*-3 PUFA might reveal themselves or compensate an athlete performing under such conditions. Hingley et al. (2017) investigated the effects of 8 weeks of DHA rich *n*-3 PUFA supplementation on high intensity cycling bouts and subsequent cycling time trial performance in 26 trained endurance athletes whilst in a state of fatigue. This study aimed to investigate the effects of *n*-3 PUFA on a different exercise modality to previous research, by recruiting a greater percentage of type II muscle fibres. Hingley et al, (2017) found no effect of fish oil on maximal cycling power (3 x 6 seconds), or on repeated Wingate cycling (6 x 30 seconds with 150 seconds active recovery). In addition, there was no observed effect of supplementation on time trial performance (5 minutes cycling as hard and as fast as possible in a fatigued state) or isometric strength of the quadriceps (determined by three maximal voluntary quadriceps contractions). This is in accordance with previous research findings (Oostenburg et

al., 1997; Buckley et al., 2009; Nieman et al., 2009 and Da Boit et al., 2015). However, oxygen consumption expressed relative to workload was significantly lower on average over the 5 min maximal cycling time trial, (compared with baseline values) in comparison to the placebo group. This difference was most notable in the first two minutes of the high intensity 5-minute cycling bout. These observations might be explained by *n*-3 PUFA and a compensatory effect when an athlete is in oxygen deficit. Whether this might be due to modulation of $\dot{V}O_2$ kinetics or amplitude is unclear (Burnley & Jones, 2007). In contrast, no changes were noted in the supplementation group during 10 minutes of steady state exercise. The fish oil dose in this particular study was relatively low dose compared to previous studies see (table 2.4)

Table 2.4 Studies that have examine the effect of n-3 PUFA on exercise performance and related physiological variables

Authors	Participants	Design	Exercise Trial	Fish oil supplementation	Supplement duration	Main effect	FO levels change in blood
Oostenbrug, et al, (1997)	24 Well trained male cyclists	Matched on W_{max}	W_{max} & prefixed workload time trial based on 70% W_{max} ~1 hour	6g fish oil daily 17.6% EPA, 12.5% DHA		2% reduction in RBC deformability. No change observed in TT or W_{max}	increase in n-3 PUFA from 5.2 - 7g/100g fatty acid in FO group
Raastad et al, (1997)	15 Well trained male soccer players	Between groups randomly assigned.	$\dot{V}O_{2\ max}$ & running performance	6.5g fish oil (2g/ day EPA and 1.30g/day DHA) or 6.5g corn oil *Participants recognized the taste of the oil therefore single blind	10 weeks	No change in $\dot{V}O_{2\ max}$ or running performance	Raised EPA (175%) and DHA(40%) in the total lipid fraction
Zebrowska et al (2004)	13 elite male cyclists	Within subject cross over	Serum NO, ADMA, $\dot{V}O_{2\ max}$	1.3g (660mg EPA and 440mg DHA)	3 weeks	Elevation in resting and exercise serum NO increased FMD and significantly improved maximal O_2 consumption and reduced HR at peak power	

Authors	Participants	Design	Exercise Trial	Fish oil supplementation	Supplement duration	Main effect	FO levels change in blood
Walser et al (2006)	7 healthy participants (male and female)	Single blind placed into independent groups	Rhythmic hand grip exercise	6g fish oil daily 3000mg EPA, 2000mg DHA	6 weeks	Enhanced brachial artery dilation and blood flow	No biomarkers mentioned in this paper
Buckley et al (2009)	25 Elite Male Australian rules football players	Parallel matched pairs	10 min SS running at 10km h. 2 x TTE tests (the second to serve as a measure of recovery)	6 x 1g sunflower oil or 1.56g DHA 0.36g EPA	5 weeks	TG, diastolic BP and HR reduced during submaximal exercise no change in TTE or recovery	Erythrocyte n-3 PUFA significantly increased
Walser et al (2008)	12 healthy participants (male and female)	Single blind placed into independent groups	20 min cycle ergometer test 10 min at low intensity and 10 min at moderate intensity	6g fish oil daily 3000mg EPA, 2000mg DHA	6 weeks	SV CO and SVR augmented during moderate intensity cycling and lowered MAP at rest	No biomarkers mentioned in this paper
Nieman et al (2009)	23 trained cyclists split into 2 groups	Random allocation to 2 groups double blind	3 days of training each day = 2 hours of submaximal cycling followed by a 10K time trial	2.4g fish oil daily (2000mg EPA and 400mg DHA)	6 weeks	No Change in immune markers or inflammatory mediators IL-1ra, IL-6 and IL-8) No change in 10K cycling performance	Plasma levels of EPA increased 311% and DHA 40%
Da Boit et al, (2015)	18 female and 19 male	random distribution	$\dot{V}O_{2\text{ peak}}$ 70% peak time trial set amount of work as quickly as possible	2g Krill oil daily (60mg EPA and 30mg DHA)	6 weeks	Increased IL-2 in stimulated PBMCs no change in IL-6, IL-4, IL-10, IL-17 and IFN- γ . No effect on time trial performance or O_2 cost of exercise or HR	Significant 75% increase in erythrocyte EPA and 21% increase in DHA fatty acid composition in Krill oil group

2.6 METHODOLOGICAL DIFFERENCES AND TIME COURSE OF SUPPLEMENTATION

Previous *n*-3 PUFA and exercise related studies vary in exercise modality, participant-training status and the dose/ duration of the supplementation protocol (see table 2.4). Supplementation, protocols vary in duration (typically between 3 – 10 weeks) and daily dosage of total *n*-3 PUFA and EPA to DHA ratio. Time course studies suggest increased EPA and DHA result in cellular peak concentrations within 4 weeks of the start of supplementation, and this is correlated to the amount of daily dose (Yaqoob et al., 2000; Healy et al., 2000). Metcalf et al. (2007), observed peak incorporation into cardiac myocytes within 30 days with a high *n*-3 PUFA dosage of (6g/day). The time course of *n*-3 PUFA incorporation in blood and adipose tissue following supplementation has been studied previously. Increases in *n*-3 PUFA incorporation in to erythrocyte membrane phospholipids have been observed within 1 week (Metherel, 2009 cited in McGlory et al., 2014). Significant Increases in *n*-3 PUFA content of adipose can take up to 12 months to incorporate (Browning, 2012, cited in McGlory et al., 2014).

McGlory et al. (2014) examined the weekly time course of *n*-3 PUFA incorporation into erythrocytes and skeletal muscle tissue in 10 healthy males who supplemented with 5g/ day of *n*-3 PUFA fish oil for 4 weeks. Venous blood samples and muscle biopsies were collected pre, post and at weekly intervals during supplementation. *N*-3 PUFA content and anabolic signalling proteins were assessed. *N*-3 PUFA content of skeletal muscle phospholipids was increased in 2 weeks and continued to increase up until week 4. This was also accompanied by alterations in anabolic signalling expression (although it should be noted this study did not have a control group to compare these increases to participants served as their own control). The

time phase required to detect skeletal muscle changes in PUFA incorporation was longer than that reported in erythrocytes (i.e. 2 weeks in skeletal muscle tissue compared to 1 week in erythrocytes), this may be due to the time course differences in the speed of tissue turnover. In addition, muscle and blood PUFA levels correlated well after 2 and 4 weeks of supplementation. Previously skeletal muscle phospholipid levels have increased ~two fold following 8 weeks of supplementation (Smith, 2011). McGlory et al. (2014), found a similar increase within 4 weeks. This may be due to the dose and high EPA concentration of the *n*-3 PUFA supplement, it was double the concentration of EPA in comparison to Smith, (2011) at 3500mg vs 1860mg/ day, DHA was 900mg. Previous studies have suggested that skeletal and heart muscle in particular incorporate high levels of DHA following supplementation with *n*-3 PUFA (Charnock et al., 1992; Owen et al., 2004). In addition, increased DHA levels may be linked to changes in muscle function, increased insulin sensitivity and modified substrate selection (Pan et al, 1995).

2.7 BIOMARKERS TO QUANTIFY N-3 PUFA STATUS

The saturation of EPA and DHA in red blood cell membranes (RBC) in relation to total fatty acid content is referred to as the omega 3 index. The RBC half-life is 4-6 times longer than in serum (Cao et al., 2006) therefore; RBC EPA and DHA content may be a more valid as a biomarker for predicting long-term PUFA intake. This has been confirmed with correlation to food frequency questionnaires (FFQ) (Sun et al 2007). It may also be a valid marker to predict myocardial tissue levels of EPA and DHA (Harris, Sands & Windsor et al 2004). Myocardial membrane EPA and DHA levels are highly correlated to erythrocyte membrane incorporation. In humans and animals, myocardial cells generally incorporate more *n*-3 PUFA (in particular DHA) than erythrocytes (McLennan, 1992; Owen, 2004).

2.8 SUMMARY

Given the proposed benefits of *n*-3 PUFA in the health context, there is increasing speculation regarding the potential benefits that *n*-3 PUFA supplementation might have in the athletic context in particular with regard to its influence on cardiovascular variables, inflammation and muscle metabolism. The review of literature presents some of the relevant *n*-3 PUFA related mechanisms that are still not completely understood. Some of the inconsistencies in previous research such as dosage/ length of supplementation protocol, modality of exercise and type of trial selected make it difficult to draw comparisons between study findings. There is a need for carefully planned systematic research to unpick *n*-3 PUFA and exercise related mechanisms.

2.9 THESIS AIMS

This main aims of this thesis were firstly to examine the acute inflammatory response to cycling. There is little research currently available on the cytokine response to different modes and intensities of cycling. As this may be an underlying target for *n*-3 PUFA action, further research is necessary to understand the cycling inflammatory model.

A further aim was to determine if an 8 week high *n*-3 PUFA low *n*-6 PUFA dietary intervention would elicit changes in oxygen consumption during submaximal cycling. Previous studies have examined *n*-3 PUFA supplementation and its effect on steady state $\dot{V}O_2$, or $\dot{V}O_{2\max}$ measurement. The current thesis will examine local skeletal muscle tissue oxygenation/ extraction (using near infrared spectroscopy) over different intensities of submaximal cycling.

Prior to examining *n*-3 PUFA and exercise related effects, a greater understanding of the use of biomarkers to substantiate participant compliance with PUFA

supplementation was required. In studies that employ athletic individuals (particularly in the field setting) the use of inexpensive, rapid analysis techniques are important. Therefore, a further aim of this thesis was to validate the measurement of fatty acids in dry blood spot samples (DBS) in relation to the current gold standard procedure of RBC separation and subsequent erythrocyte fatty acid analysis. The experimental studies in this thesis were designed in order to answer the following research questions

- 1) Does 1-hour of pre-loaded submaximal cycling result in a different cytokine response (post exercise) compared to high intensity interval cycling?
- 2) Is the fingertip whole blood DBS method of determining an individual's fatty acid status a valid method, in relation to isolated erythrocyte analysis (considered gold standard)?
- 3) Does high *n*-3 PUFA supplementation, coupled with dietary modification to lower *n*-6 PUFA intake, successfully increase *n*-3 and decrease *n*-6 PUFA incorporation into erythrocyte phospholipids?
- 4) Does 3) affect $\dot{V}O_{2\text{ peak}}$ performance?
- 5) Does 3) result in changes in $\dot{V}O_{2\text{ peak}}$, tissue saturation Index (TSI %) or deoxy-HHb during cycling at four submaximal intensities?

CHAPTER 3 THE CYTOKINE RESPONSE TO TWO
CYCLING MODALITIES: 1) PRE-LOADED PERFORMANCE
TRIAL AND 2) HIGH INTENSITY INTERVAL TRIAL.

INTRODUCTION

Cytokines are a group of proteins that mediate the inflammatory response to infection and injury. Cytokine production is also mediated by a number of other factors including exercise. Exercise induced changes in cytokine levels play an important role in post exercise immune function. During exercise, cytokine changes may be triggered by a number of factors such as muscle and tissue damage (Nieman et al., 2005), circulating stress hormones, carbohydrate depletion and oxidative stress (Cannon, 2000). Cytokines are classified as either pro or anti-inflammatory. Anti-inflammatory cytokines such as, IL-4, IL-10, MCP-1, IL-1RA inhibit the production of pro inflammatory cytokines such as IL-1 β and TNF- α . IL-6 can act as both a pro - and anti-inflammatory cytokine (Peake et al, 2005; Hirose et al, 2004). IL-6 increases acutely after exercise and initiates changes in pro-inflammatory and anti-inflammatory cytokine production post exercise (Nieman et al., 2001; Suzuki et al, 2000). For example IL-10 and IL-1ra are thought to be anti-inflammatory cytokines that may counteract the inflammatory response (Ostrowski, et al 1999; Suzuki, 2000), in order to keep the body in homeostasis.

To our knowledge, there has been no attempt to directly compare acute cytokine changes in response to cycling modes that differ in intensity and duration. Much of the research has investigated long duration endurance exercise such as marathon and ultra-marathon events (Nieman et al., 2003; Ostrowski et al., 1999), down-hill running and eccentric exercise (Peake, 2005). These events produce extensive muscle damage likely to cause large changes in the cytokine/ immune response. Although it is expected that cycling may produce a lesser magnitude of inflammatory response (due to the low impact nature of cycling), athletes may still be prone to pro-inflammatory cytokine elevation when engaging in regular moderate training or during chronic training periods. Reducing exercise-induced inflammation for cyclists is important for a number of reasons. Although inflammation is part of the normal

human physiological response to infection and Injury, when it occurs in an uncontrolled or inappropriate manner (for example in response to heavy exercise loads, long duration exercise or increased frequency of training), damage to tissues, loss of function and disease can occur (Calder 2006).

Few studies have directly compared differing exercise loads for the same mode of exercise. Brenner et al. (1999) examined the effect of four different types of exercise on inflammatory biomarkers in the same participant group. Out of the four exercise conditions which included, all out (5 minutes of cycle ergometer exercise 90% $\dot{V}O_{2\max}$), circuit training (5 types of exercise 3 sets of 10 at 60 -70% RM), long (two hours of cycling at 60 - 65% $\dot{V}O_{2\max}$), and control, the long exercise condition resulted in significant increases in plasma IL-6 and TNF α . Peake et al. (2005), examined plasma cytokine changes in relation to exercise intensity and muscle damage in well-trained male runners and triathletes. The exercise conditions included a 1 hour run at 60% $\dot{V}O_{2\max}$, a 1-hour run at 80% $\dot{V}O_{2\max}$, and downhill running (10% gradient for 45 minutes). High intensity running (80% $\dot{V}O_{2\max}$) had a greater effect than 1 hour moderate running or downhill running on plasma levels of IL-1ra, IL10, IL-12, and PGE₂. The two mentioned studies appear to indicate that inflammatory cytokines are most likely to be elevated following long intense exercise, rather than short and moderate intensity exercise.

In contrast Cox (2007), observed no significant difference in the inflammatory response to 60min running at 65% $\dot{V}O_{2\max}$ compared to 6 x 3 minute intervals at 90% $\dot{V}O_{2\max}$ in well trained male distance runners. An increase in IL-6 and IL-1ra was observed post and at 1-hour post both protocols however, there was no significant difference in the magnitude of change pre to post exercise or between the two trials.

Few studies have examined the cytokine response to cycling, or modes of cycling that differ in intensity and duration. The majority of previous research focuses on modes of exercise that elicit muscle damage. As the focus of this thesis is on endurance cycling and the effects of fish oil on physiological mechanisms (some of which are related to exercise induced inflammation), it would be important to begin to substantiate the magnitude of the cycling induced inflammatory response.

In most cases, cytokine concentration is analysed in blood plasma, which can become invasive due to the number of repeated samples required. Drawing samples from the antecubital vein is reliant on the availability of phlebotomy-trained personnel and the costs associated with this. Such factors have resulted in the development of different assays for assessing cytokine concentration that are less invasive and do not require trained individuals to collect samples. One such method includes the analysis of saliva samples (Nishanian et al., 1988). The relationship between saliva and plasma IL-1 β is elusive. IL-1 β levels are known to be much higher in saliva than plasma (Brailo, 2012; Llamas Moya, 2006) which might appear to be of benefit due to many plasma derived cytokines being below the detection limits of high sensitivity assays (Wong, 2008). Although the question why this is the case should be carefully considered. It is possible that salivary cytokine levels reflect local inflammatory status in the mouth such as periodontitis or upper respiratory tract infection (Gaphor et al, 2014). Plasma IL-1 β concentrations may be lower, but may reflect systemic inflammation more accurately. Few studies have examined the relationship between IL-1 β plasma and saliva assays or looked at the level of agreement between them. Williamson, (2011) observed no significant correlations between cytokine plasma samples and filter paper saliva samples (including IL-1 β).

Therefore, the aim of this study was to examine the effect of two different cycling trials on well-known plasma inflammatory and anti-inflammatory markers. More specifically the study aims to examine firstly if any significant cytokine changes

occur in response to acute cycling exercise (and if so if this change differs, in response to a 1-hour submaximal-cycling trial compared to a high intensity interval trial). A secondary aim of this study was to examine the relationship and level of agreement between plasma and salivary assays for determining IL-1 β concentration.

METHODS

Subjects

Fourteen moderately healthy male competitive road cyclists and triathletes were recruited, and ten participated in the study. Participants were recruited by email from cycling and triathlon clubs in London and the South East. Moderate training status was defined as $2 \geq$ hours of continuous cycling per week and for the past six months. Participants were excluded from the study if they had any incidence of illness or recent injury, were taking any medication, had a history of hyperlipidaemia, hypertension, and diabetes mellitus or were regularly consuming daily fish oil supplements or fish containing meals ($2 \geq$ servings per week). The study was approved by the University of Kent Research Advisory Group (REAG). The Participant's physical characteristics are presented in Table 3.1

Table 3.1 Participant's physical characteristics

Physical characteristics	Mean \pm SD
Age (yrs)	39 \pm 11
Height (cm)	176.9 \pm 5.9
Weight (kg)	75.5 \pm 9.6
$\dot{V}O_{2\text{ peak}}$ (ml/ kg/ min)	57.6 \pm 7.3
Peak power (W)	328 \pm 48

Experimental design

Participants reported to the laboratory on four occasions over a four-week period. All participants completed an informed consent form and a medical questionnaire. The first test was used to determine cardiorespiratory fitness ($\dot{V}O_{2\text{ peak}}$) and peak power output. The second test was used to familiarise the participants with the two cycling protocols. Subsequently, subjects returned to the laboratory for two separate visits to complete the experimental testing, which consisted of 1) a pre-loaded performance trial and 2) a high intensity interval trial. The two experimental tests were performed in a random order over visits three and four. Participants attended each visit at the same time of day. Each laboratory session was separated by at least 1 week. Participants were instructed to arrive in a rested and euhydrated state, having abstained from alcohol, caffeine and exercise training for the previous 24 hours, and having fasted for 2 hours with water consumption permitted ad libitum.

Determination of $\dot{V}O_{2\text{ peak}}$

On arrival at the laboratory, participant's height and weight was recorded. Testing was performed on an electronically braked cycle ergometer (SRM Schoberer, Germany). The bike was adjusted and the settings recorded so the same bike set up could be reproduced for each trial. Participants commenced a 10 minute warm up at 100 W, immediately followed by a graded exercise protocol starting at 150 W. Power increased by 20 W each minute until volitional exhaustion, as employed by Nieman et al. (2009). Participants were instructed to maintain their preferred cadence throughout the test, which was terminated when pedal cadence dropped more than 10 rpm below the chosen cadence for 10 seconds (despite strong verbal encouragement). Breath by breath analysis of oxygen uptake was determined using

a (Quark B2 analyser and associated software). Data was reduced to 10-second average $\dot{V}O_2$. $\dot{V}O_{2\text{ peak}}$ was determined as the highest $\dot{V}O_2$ over a 60-second period. Heart rate (HR) (Polar RS400 and wear link) and rating of perceived exertion (RPE) (Borg, 1970) was recorded at 1-minute intervals and power output was sampled every 0.5 seconds. Peak power was determined as the highest wattage recorded over a 60-second period. A fingertip capillary blood sample was obtained at rest and immediately post test to determine whole blood glucose and lactate levels (Biosin C-line EKF Diagnostic).

Standardisation of diet

Participants were directed to keep a 24-hour food diary prior to their second laboratory visit, and were asked to use this record to repeat the same food and fluid intake in the 24 hours prior to visits 2, 3 and 4.

Pre-loaded Performance trial (TT)

Participants commenced a 10 minute warm up at 100 W, on a cycle ergometer (SRM Schoberer, Germany) followed by a 2-minute rest period. Participants then commenced a 1-hour pre-loaded performance trial. The cycle ergometer was set to hyperbolic mode for the first 45 minutes of the test. Participants cycled at 70% of their maximum peak power output (determined in visit 1) at their preferred cadence. The cycle ergometer was then switched to linear mode for the final 15 minutes of the test (in linear mode, resistance is determined automatically in response to cadence so power output could be determined by the participant). Participants were instructed to perform as much work as possible during the final 15 minutes of the test.

High intensity interval trial (INT)

Participants commenced a 10 minute warm up at 100 W, on a cycle ergometer (SRM Schoberer, Germany) followed by a 2-minute rest period. They then completed up to 12 x 1-minute cycling intervals (or until volitional exhaustion whichever came first) at their maximum peak power output (determined in visit 1) at their preferred cadence. Each interval was separated with 75 seconds of cycling at 100 W. The 1-hour pre-loaded time trial and interval trial were selected as they represent typical training sessions that cyclists complete on a regular basis.

Sample collection and analysis

Blood samples

Immediately pre and post both the pre-loaded performance trial and the interval trial, a 7ml venous blood sample was collected by a trained phlebotomist using standard venepuncture procedure. Whole blood was collected directly into EDTA collection tubes. Plasma was separated by centrifugation at 10,000 x g for 10 minutes at 5°C. Clear plasma was pipetted into separate 3ml aliquots and stored frozen at -80°C until later analysis.

Plasma cytokine concentrations were determined using a cytokine 1 high sensitivity array for use with an evidence investigator biochip analyser (Randox laboratories Ltd UK). According to the manufacturer's instructions, all samples were analysed in duplicate. The cytokine 1 high sensitivity array simultaneously measured the following 11 parameters from a single sample aliquot: IL-2, IL-4, IL-6, IL-8, IL-10, IFN- γ , TNF- α , IL-1 α , IL-1 β , MCP-1 and VEGF. The lowest concentration for each cytokine (in accordance with the assay manual with imprecision of $\leq 20\%$ for 20 replicates), was recorded when results were below the detection limit of the assay kit.

Saliva samples

Unstimulated saliva samples (~0.5ml) were collected pre and post exercise, directly into sterile conical collection tubes (Fisher Scientific). Participants were asked to allow saliva to collect in their mouth and over time expel it into the collection tube. Participants were asked to avoid actively bringing saliva, sputum or phlegm up from the back of the throat. Samples were immediately centrifuged at 10,000g for 10 minutes at 5°C. Clear saliva was pipetted into aliquots and stored frozen at -80°C until later analysis.

Salivary IL-1 β concentrations were determined using commercially available IL-1 β immunoassay kit (Salametrics, PA, USA) according to the manufacturer's instructions all samples were analysed in duplicate. Saliva samples were checked for osmolality (S_{osm}) by freezing point depression using an osmometer.

Statistical analysis

Determination of plasma cytokine concentrations pre & post the performance trial (TT) and the high intensity Interval trial (INT).

Blood plasma concentrations of the following cytokines: IL-2, IL-6, IL-8, IFN- γ , TNF- α , IL-1 β , MCP-1, VEGF, IL-4, IL-10, IL-1R α , were screened in accordance with analysis of variance (ANOVA) assumptions. There were no outliers as assessed by examination of studentized residuals for values greater than ± 3 . Cytokine concentration values were normally distributed for IL-8 and MCP-1 ($p > 0.05$), as assessed by Shapiro Wilk test of normality. IL-1 α , IL-1 β , IL-2, IL-6, IL-10, values were transformed using Log-10. IL-4 and IFN- γ were reciprocal transformed. Cytokine responses to (TT) and (INT) were assessed using a 2 x 2 time (pre/ post)

x trial (TT/ INT) ANOVA. Post hoc Bonferroni-corrected paired samples t -tests were carried out to identify the location of differences when a main effect of trial or an interaction between time and trial was observed. All analysis were completed using the statistical package IBM SPSS statistics version 24, with significance accepted at $p < 0.05$.

Comparison of plasma and saliva assays in order to determine IL-1 β cytokine concentrations

A Pearson's product moment correlation was carried out to assess the relationship between IL1- β values for 1) blood plasma & saliva and 2) blood plasma & saliva expressed as concentration: osmolality for the following conditions a) pre-TT b) post-TT c) pre-INT d) post-INT.

A Pearson's product moment correlation was carried out to assess the relationship between the magnitude of change (percentage change pre to post exercise relative to 100) in IL1- β for TT and INT. Percentage Changes were correlated for the following assays 1) blood plasma & saliva and 2) blood plasma & saliva expressed as concentration: osmolality. Data was not normally distributed as assessed by Shapiro-Wilk's test ($p > 0.05$); therefore, a log 10 transformation was applied.

The level of agreement between assays (for percentage change values) were examined using Bland and Altman agreement plots (Bland & Altman, 1986)

RESULTS

Table 3.2 presents plasma Cytokine concentrations pre and post the preloaded time trial and interval trial (mean \pm standard deviation). The following is a summary of the two-way ANOVA results for each cytokine.

IL-1 α

There was no significant main effect of time (pre/ post), $F(1, 9) = 1.960$, $p = 0.195$, partial $\eta^2 = 0.179$, or cycling trial, $F(1, 9) = 0.051$, $p = 0.826$, partial $\eta^2 = 0.006$, and no significant interaction between cycling trial and time on IL-1 α concentration, $F(1, 9) = 2.684$, $p = 0.136$, $\eta^2 = 0.230$.

IL-1 β

There was no significant main effect of time (pre/ post), $F(1, 9) = 0.091$, $p = 0.770$, partial $\eta^2 = 0.01$, or cycling trial, $F(1, 9) = 1.686$, $p = 0.226$, partial $\eta^2 = 0.158$ on IL-1 β concentration. There was a significant interaction between cycling trial and time, $F(1, 9) = 8.058$, $p = 0.019$, $\eta^2 = 0.472$. In follow up to the interaction, Bonferroni corrected post - hoc paired t -tests showed that IL-1 β concentration did not significantly differ pre to post exercise in the time trial ($p = 0.636$), or pre to post exercise in the interval trial ($p = 0.276$). Furthermore there was no significant difference in IL-1 β between trials pre-exercise ($p = 0.516$), or between trials post exercise ($p = 0.248$).

IL- 2

There was no significant main effect of time (pre/ post), $F(1, 9) = 0.911$, $p = 0.365$, partial $\eta^2 = 0.092$, or cycling trial, $F(1, 9) = 1.291$, $p = 0.285$, partial $\eta^2 = 0.125$, and no significant interaction between cycling trial and time on IL-2 concentration, $F(1, 9) = 0.130$, $p = 0.727$, $\eta^2 = 0.014$.

IL-4

There was a significant main effect of time (pre/ post). IL-4 concentration for both trials combined was higher on average post, compared to pre exercise, $F(1, 9) = 9.654$, $p = 0.013$, partial $\eta^2 = 0.518$. There was no significant main effect of cycling trial, $F(1, 9) = 0.022$, $p = 0.886$, partial $\eta^2 = 0.002$. Furthermore there was no significant interaction between cycling trial and time on IL-4 concentration, $F(1, 9) = 3.754$, $p = 0.085$, $\eta^2 = 0.294$.

IL-6

There was a significant main effect of time (pre/ post), $F(1, 9) = 25.823$, $p = 0.001$, partial $\eta^2 = 0.742$, and cycling trial, $F(1, 9) = 10.80$, $p = 0.009$, partial $\eta^2 = 0.545$. There was a significant interaction between cycling trial and time, $F(1, 9) = 8.875$, $p = 0.015$, $\eta^2 = 0.497$. Bonferroni corrected post - hoc paired t – tests showed a significant pre to post increase in IL-6 concentration in both the time trial, ($p = 0.008$) and the interval trial ($p = 0.004$). Furthermore, there was no significant difference between trials pre exercise ($p = 1.00$) however there was a significant difference between trials post exercise, the IL-6 response was significantly higher post the time trial compared to post the interval trial ($p = 0.016$).

IL-8

There was no significant main effect of time (pre/ post), $F(1, 9) = 0.482$, $p = 0.505$, partial $\eta^2 = 0.051$. There was a significant main effect of cycling trial. IL-8 concentrations pre and post exercise combined were higher on average for the time trial in comparison to the interval trial, $F(1, 9) = 10.192$, $p = 0.011$, partial $\eta^2 = 0.531$. However, there was no significant interaction between cycling trial and time, $F(1, 9) = 0.039$, $p = 0.848$, $\eta^2 = 0.004$.

IL-10

There was no significant main effect of time (pre/ post), $F(1, 9) = 3.201$, $p = 0.107$, partial $\eta^2 = 0.262$, or cycling trial, $F(1, 9) = 0.961$, $p = 0.353$, partial $\eta^2 = 0.096$ and no significant interaction between cycling trial and time, $F(1, 9) = 0.464$, $p = 0.513$, $\eta^2 = 0.049$.

IFN- γ

There was no significant main effect of time (pre/ post), $F(1, 9) = 0.922$, $p = 0.362$, partial $\eta^2 = 0.093$, or cycling trial, $F(1, 9) = 0.430$, $p = 0.528$, partial $\eta^2 = 0.046$, and no significant interaction between cycling trial and time, $F(1, 9) = 1.143$, $p = 0.313$, $\eta^2 = 0.113$.

TNF- α

There was no significant main effect of time (pre/ post), $F(1, 9) = 0.710$, $p = 0.421$, partial $\eta^2 = 0.073$, or cycling trial, $F(1, 9) = 1.347$, $p = 0.276$, partial $\eta^2 = 0.130$ and no significant interaction between cycling trial and time, $F(1, 9) = 1.116$, $p = 0.318$, $\eta^2 = 0.110$.

MCP-1

There was a significant main effect of time (pre/ post), $F(1, 9) = 18.76$, $p = 0.002$, partial $\eta^2 = 0.676$ and no significant main effect of cycling trial, $F(1, 9) = 0.515$, $p = 0.491$, partial $\eta^2 = 0.054$. There was a significant interaction between cycling trial and time, $F(1, 9) = 13.74$, $p = 0.005$, $\eta^2 = 0.604$. Bonferroni corrected post – hoc paired t -tests showed a significant pre to post exercise increase in MCP-1 in the time trial ($p < 0.001$). There was no change in MCP – 1 concentration pre to post the interval trial ($p = 1.00$). Furthermore, there was no significant difference in MCP-1 concentration between trials pre exercise ($p = 1.00$) or between trials post exercise ($p = 0.21$).

VEGF

There was a significant main effect of time (pre/ post), VEGF concentration for both trials on average were higher pre exercise compared to post, $F(1, 9) = 8.740$, $p = 0.016$, partial $\eta^2 = 0.493$. There was no significant main effect of cycling trial, $F(1, 9) = 2.767$, $p = 0.131$, partial $\eta^2 = 0.235$, and no significant interaction between cycling trial and time, $F(1, 9) = 0.260$, $p = 0.622$, $\eta^2 = 0.028$.

Table 3.2. Plasma Cytokine concentration data pre and post the pre-loaded time trial and interval trial.

Cytokine	Trial	Mean \pm SD	95% CI		ANOVA Main effects & Interaction <i>Trial (TT/INT)</i> <i>Time (Pre/Post)</i> <i>Trial * time</i>	partial η^2
			Lower	Upper		
IL-1 α (N = 10)	Pre TT	0.58 \pm 0.74	0.05	1.11	$F(1, 9) = 0.051, p = 0.826$	0.006
	Post TT	0.46 \pm 0.47	0.12	0.796	$F(1, 9) = 1.960, p = 0.195$	0.179
	Pre INT	0.40 \pm 0.42	0.10	0.71	$F(1, 9) = 2.684, p = 0.136$	0.230
	Post INT	0.50 \pm 0.52	0.13	1.87		
IL-1 β (N = 10)	Pre TT	3.43 \pm 3.92	0.62	6.24	$F(1, 9) = 1.686, p = 0.226$	0.158
	Post TT	2.76 \pm 3.74	0.88	5.44	$F(1, 9) = 0.091, p = 0.770$	0.010
	Pre INT	2.69 \pm 3.12	0.46	4.92	$F(1, 9) = 8.058, p = 0.019^*$	0.472
	Post INT	3.60 \pm 4.06	0.69	6.50		
IL-2 (N = 10)	Pre TT	6.14 \pm 7.62	0.70	11.59	$F(1, 9) = 1.291, p = 0.285$	0.125
	Post TT	5.45 \pm 6.67	0.67	10.22	$F(1, 9) = 0.911, p = 0.365$	0.092
	Pre INT	4.37 \pm 6.01	0.08	8.68	$F(1, 9) = 0.130, p = 0.727$	0.014
	Post INT	5.40 \pm 6.91	0.45	10.35		
IL-4 (N = 10)	Pre TT	5.08 \pm 5.20	1.36	8.80	$F(1, 9) = 0.022, p = 0.886$	0.002
	Post TT	5.29 \pm 5.59	1.29	9.29	$F(1, 9) = 9.654, p = 0.013^*$	0.518
	Pre INT	4.11 \pm 3.45	1.64	6.58	$F(1, 9) = 3.754, p = 0.085$	0.294
	Post INT	4.90 \pm 4.13	1.95	7.85		
IL-6 (N = 10)	Pre TT	2.56 \pm 3.30	0.20	4.92	$F(1, 9) = 10.80, p = 0.009^{**}$	0.545
	Post TT	6.68 \pm 4.48	3.48	9.88	$F(1, 9) = 25.82, p = 0.001^{**}$	0.742
	Pre INT	2.16 \pm 2.05	0.69	3.63	$F(1, 9) = 8.875, p = 0.015^*$	0.497
	Post INT	2.95 \pm 2.24	1.35	4.55		
IL-8 (N = 10)	Pre TT	7.12 \pm 4.29	4.05	10.19	$F(1, 9) = 10.192, p = 0.011^*$	0.531
	Post TT	7.31 \pm 4.77	3.90	10.73	$F(1, 9) = 0.482, p = 0.505$	0.051
	Pre INT	4.29 \pm 2.42	2.56	6.02	$F(1, 9) = 0.039, p = 0.848$	0.004
	Post INT	4.71 \pm 2.53	2.90	6.52		
IL-10 (N = 10)	Pre TT	2.02 \pm 2.39	0.31	3.72	$F(1, 9) = 0.961, p = 0.353$	0.096
	Post TT	3.06 \pm 1.57	0.94	3.19	$F(1, 9) = 3.201, p = 0.107$	0.262
	Pre INT	1.08 \pm 1.09	0.30	1.86	$F(1, 9) = 0.464, p = 0.513$	0.049
	Post INT	2.57 \pm 3.47	0.09	5.06		
IFN- γ (N = 4)	Pre TT	0.73 \pm 0.57	0.32	1.14	$F(1, 9) = 0.430, p = 0.528$	0.046
	Post TT	0.60 \pm 0.29	0.39	0.80	$F(1, 9) = 0.922, p = 0.362$	0.093
	Pre INT	0.68 \pm 0.39	0.41	0.96	$F(1, 9) = 1.143, p = 0.313$	0.113
	Post INT	0.75 \pm 0.51	0.38	1.11		
TNF α (N = 10)	Pre TT	3.49 \pm 2.70	1.56	5.42	$F(1, 9) = 1.347, p = 0.276$	0.130
	Post TT	3.26 \pm 1.77	1.99	4.53	$F(1, 9) = 0.710, p = 0.421$	0.073
	Pre INT	3.06 \pm 2.46	1.30	4.82	$F(1, 9) = 1.116, p = 0.318$	0.110
	Post INT	3.05 \pm 1.83	1.74	4.36		
MCP-1 (N = 10)	Pre TT	74.69 \pm 25.34	56.57	92.82	$F(1, 9) = 0.515, p = 0.491$	0.054
	Post TT	102.01 \pm 36.75	75.72	128.31	$F(1, 9) = 18.762, p = 0.002^{**}$	0.676
	Pre INT	84.24 \pm 28.32	63.98	104.50	$F(1, 9) = 13.737, p = 0.005^{**}$	0.604
	Post INT	79.33 \pm 15.41	68.30	90.35		
VEGF (N = 10)	Pre TT	26.45 \pm 14.91	15.78	37.11	$F(1, 9) = 2.767, p = 0.131$	0.235
	Post TT	21.63 \pm 12.65	12.59	30.68	$F(1, 9) = 8.740, p = 0.016^*$	0.493
	Pre INT	19.40 \pm 4.97	15.84	22.95	$F(1, 9) = 0.260, p = 0.622$	0.028
	Post INT	16.94 \pm 3.63	14.34	19.54		

Data is reported as mean \pm SD. Values are in pg. / mL, TT = pre-loaded time trial, INT = interval trial, $p < 0.05^*$, $p < 0.01^{**}$.

Table 3.3. Pearson's product moment correlation to determine the relationship between IL-1 β cytokine concentration in plasma and saliva (and saliva expressed as concentration : osmolality), pre and post a pre-loaded time trial and an Interval trial.

Trial	<i>N</i>	Plasma IL-1 β <i>Mean \pm SD</i>	Saliva IL-1 β <i>Mean \pm SD</i>	Saliva C:O <i>Mean \pm SD</i>	PLA & SAL Pearson's <i>r</i>	PLA & SAL Sig (2 tailed)	PLA & C:O Pearson's <i>r</i>	PLA & C:O Sig (2 tailed)
TT pre	9	2.93 \pm 3.82	256.90 \pm 199.22	4.23 \pm 2.58	0.498 (-0.495 – 0.874)	0.173	0.458 (-0.269 – 0.863)	0.215
TT post	9	2.68 \pm 3.95	292.64 \pm 260.47	3.62 \pm 2.55	0.807* (0.570 – 0.967)	0.009*	0.781* (0.504 – 0.979)	0.013*
INT pre	9	2.44 \pm 3.21	270.70 \pm 308.98	4.30 \pm 3.55	0.432 (-0.156 – 0.783)	0.245	0.297 (-0.362 – 0.708)	0.437
INT post	9	3.38 \pm 4.25	233.43 \pm 237.64	3.34 \pm 2.54	0.245 (-0.611 – 0.718)	0.525	0.150 (-0.562 – 0.679)	0.701

Data is displayed as mean \pm SD, IL-1 β concentration is expressed as pg/ mL, * $p = <0.05$, percentile bootstrap 95% CI's reported in brackets

TT = pre-loaded time trial, INT = interval trial, PLA = plasma, SAL = saliva, CO = IL-1 β expressed as concentration: osmolality

Data was transformed Log 10

There was not a significant relationship between plasma and saliva IL-1 β concentration pre the pre-loaded time trial, however there was a significant relationship between them post. When saliva data was expressed as concentration : osmolality, similar results were observed pre and post the pre-loaded time trial. There was no significant relationship between measures pre and post the Interval trial (see table 3.3).

Table 3.4. Pearson's product moment correlation to determine the relationship between plasma and saliva (and saliva expressed as concentration : osmolality) percentage change (relative to 100) in IL-1 β concentration pre to post the pre-loaded time trial and interval trial.

Trial	<i>N</i>	Plasma IL-1 β <i>Mean \pm SD</i>	Saliva IL-1 β <i>Mean \pm SD</i>	Saliva C:O <i>Mean \pm SD</i>	PLA & SAL Pearson's <i>r</i>	PLA & SAL Sig (2 tailed)	PLA & C:O Pearson's <i>r</i>	PLA & C:O Sig (2 tailed)
TT % change pre – post	9	89.40 \pm 29.11	116.89 \pm 48.86	91.91 \pm 36.04	0.044 (-0.589 – 0.830)	0.911	0.010 (-0.537 – 0.688)	0.981
INT % change pre – post	9	173.77 \pm 148.57	116.36 \pm 87.96	107.83 \pm 72.19	0.511 (-0.659 – 0.882)	0.160	0.677* (-0.278 – 0.957)	0.045*

Data is displayed as mean \pm SD, IL-1 β values are expressed in % change in pg/ mL pre to post exercise trial relative to 100, * $p = <0.05$, percentile bootstrap 95% CI's reported in brackets

TT = pre-loaded time trial, INT = interval trial, PLA = plasma, SAL = saliva, CO = IL-1 β expressed as concentration: osmolality

Data was transformed Log 10

There was no significant relationship between plasma and saliva IL-1 β percentage change (relative to 100), pre to post the pre-loaded time trial or pre to post the Interval trial. However when saliva data was expressed as concentration : osmolality there was a significant relationship between plasma and saliva percentage change relative to 100 for the interval trial.

Table 3.5 Bland and Altman level of agreement between plasma and saliva (and saliva expressed as concentration : osmolality) percentage change (relative to 100) in IL-1 β concentration pre to post the pre-loaded time trial and interval trial.

Trial	(N)	Plasma IL-1 β <i>Mean \pm SD</i>	Saliva IL-1 β <i>Mean \pm SD</i>	Mean % change (SAL+PLA)/2	Mean diff SAL - PLA	-1.96s	+1.96s
TT % change pre – post	9	89.40 \pm 29.11	116.89 \pm 48.86	103.15 \pm 30.94	-27.49 \pm 51.39	-128.21	73.23
INT % change pre – post	9	173.77 \pm 148.57	116.36 \pm 87.96	119.43 \pm 40.88	6.13 \pm 103.88	-197.48	209.73
<i>Saliva data expressed as concentration: osmolality</i>							
TT % change pre – post	9	89.40 \pm 29.11	91.91 \pm 36.04	90.65 \pm 25.03	-2.51 \pm 42.28	-85.37	80.35
INT % change pre – post	9	173.77 \pm 148.57	107.83 \pm 72.19	14.74 \pm 47.93	14.66 \pm 81.20	-144.48	173.81

Data is displayed as mean \pm SD, IL-1 β values are expressed in % change in pg/ mL pre to post exercise trial relative to 100,
TT = pre-loaded time trial, INT = interval trial, PLA = plasma, SAL = saliva.
Data was transformed Log 10

Bland and Altman analysis indicated poor agreement between plasma and saliva IL-1 β for % change percentage change (relative to 100) in IL-1 β concentration pre to post the pre-loaded time trial and interval trial (see table 3.5 and figure 3.1).

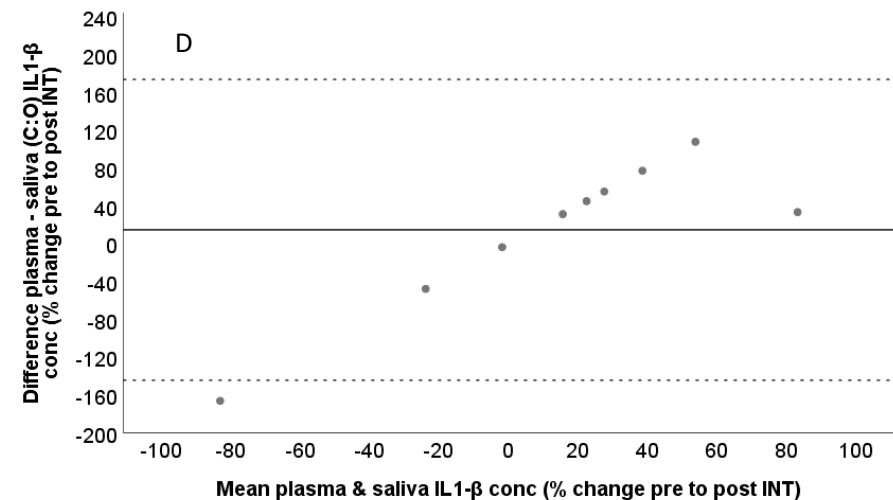
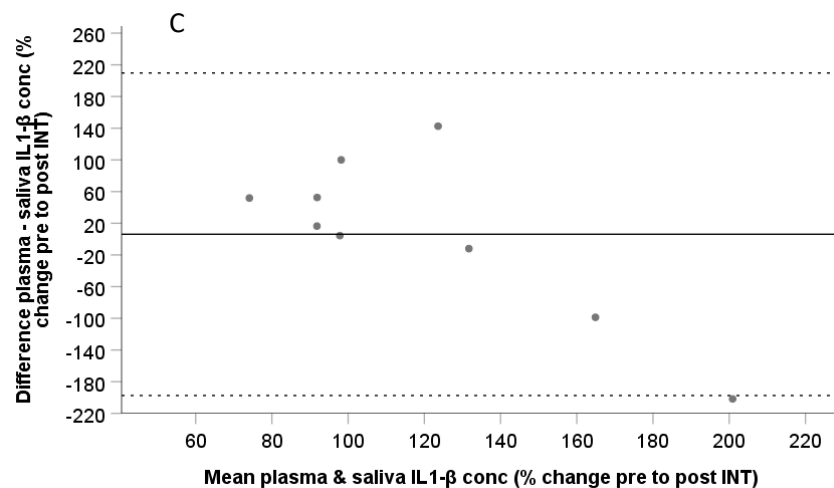
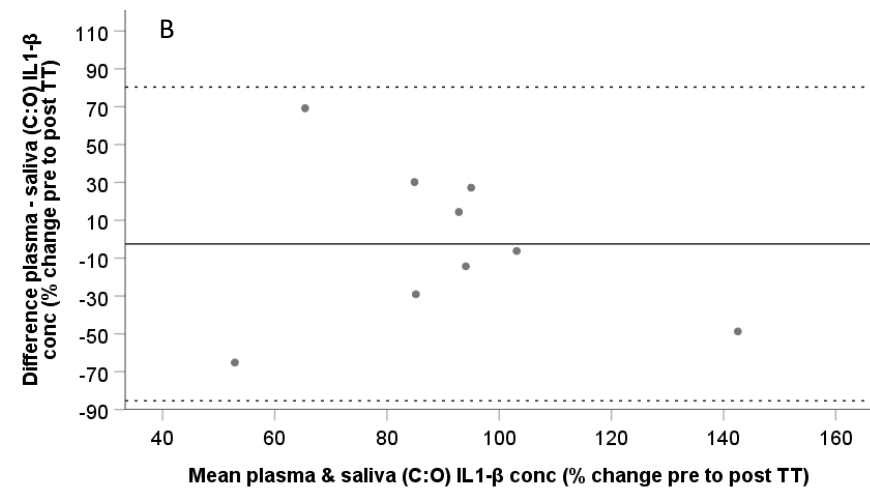
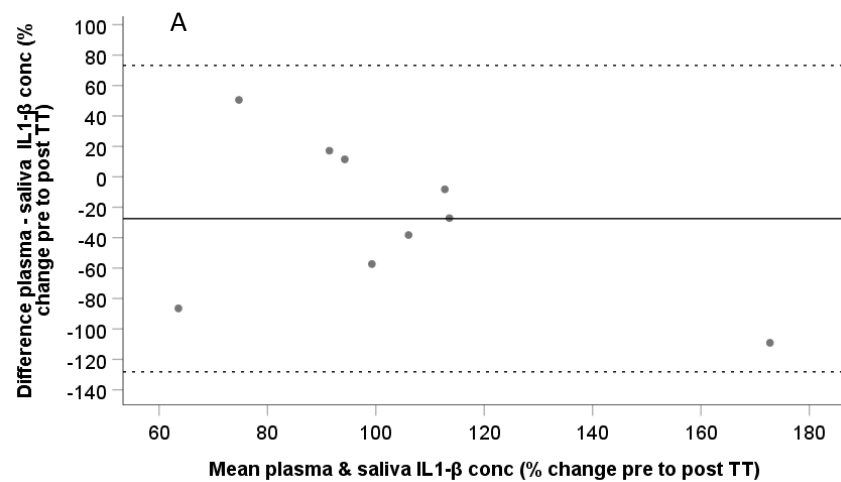


Figure 3.1. Bland and Altman level of agreement between plasma and saliva percentage change (relative to 100) in IL1-β concentration pre to post: (A) pre-loaded time trial (TT), (B) pre-loaded time trial saliva expressed as concentration osmolality C:O, (C) Interval trial (INT), (D) interval trial C:O

DISCUSSION

The aim of the present study was to examine the influence of two different cycling protocols (a pre-loaded time trial and a high intensity interval trial) on changes in plasma concentrations of 11 cytokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IFN- γ , TNF- α , MCP-1 and VEGF). The main findings of this study demonstrate that IL-6 concentration significantly increased post both cycling trials compared to pre, and the increase following the preloaded time trial was significantly greater than the interval trial. When expressed as percentage change, there was a 161% increase in IL-6 following the time trial compared to a 37% increase following the interval trial. Secondary findings of this study show that there was a main effect of time for IL-4, MCP-1 and VEGF. IL-4 and MCP-1 concentration significantly increased and VEGF concentration significantly decreased for both trials combined post exercise, these changes were not different between trials. No changes in concentrations of IL-1 α , IL-1 β , IL-2, IL-8, IL-10, IFN- γ , or TNF α were observed pre to post exercise, or between the two trials.

The elevation in plasma IL-6 concentration observed post both trials and in particular, post the 1-hour pre-loaded time trial is consistent with the findings of others researchers (Cox, 2007; Suzuki, 2000). Brenner et al. (1999), reported the IL-6 response was more marked post 2 hours of cycling at 60%, $\dot{V}O_{2\text{ max}}$ compared to five minutes of high intensity cycling or circuit training. In Brenner et al. (1999), the increase in IL-6 was five to six fold greater than baseline levels. In the current study the increase was not as high as this (two to three fold that of baseline levels); this might be related to the difference in exercise duration between the two studies. Scott (2011), found plasma concentrations of IL-6 peaked immediately following an acute bout of treadmill running at 75% $\dot{V}O_{2\text{ max}}$, and remained elevated for 30

minutes post exercise. In marathon running, IL-6 has been observed to peak immediately following the race (Ostrowski et al., 1999). Smith et al. (2000) reported increased IL-6 concentrations in untrained college males at 6, 12, 24 and 48 hours post eccentric bench press and leg curl exercise. Toft et al. (2002) found IL-6 significantly increased following 60 min of eccentric lower limb exercise in young and elderly untrained individuals. The elevated IL-6 response to exercise is well documented, IL-6 is considered to be multifunctional, displaying several pro-inflammatory properties however, it also has anti-inflammatory properties (Smith et al., 2000; Pedersen, 2000, 2004). IL-6 appears in the circulation preceding other cytokines, and is thought to be responsible for the trigger of other anti-inflammatory cytokines including, IL-4 (Cox, 2007), IL-10 and IL-1ra (Smith, 2000; Suzuki, 2002). IL-6 is a cytokine released locally in the muscle also described as a myokine (Pederson, 2004). Steensberg et al. (2000), measured femoral arterial-venous differences across an exercising leg compared to a resting leg, and reported that IL-6 is only released in the exercising limb. In addition, isolated nuclei from muscle biopsy samples show the IL-6 transcription rate increases following the onset of exercise (Keller et al. 2001). IL-6 increases exponentially with exercise and is related to exercise duration, intensity and the mass of muscle recruited (Febbraio & Pederson, 2002). Therefore, there is evidence to support that, IL-6 production is associated with the contracting skeletal muscle, and its production is independent of muscle damage (Gokhale, 2007). In addition, IL-6 production has been associated with muscle glycogen depletion; this may explain the more pronounced increase in IL-6 observed post the longer duration 1 hour time trial compared to the interval trial in the current study. In support of this, Steesberg et al. (2001) reported that IL-6 concentration is elevated earlier in a glycogen depleted leg (detected 60 min following the onset of exercise), compared to a non-glycogen depleted leg (detected 120 minutes following the onset of exercise). Therefore, muscle glycogen

status is considered a stimulus for the transcription of the IL-6 gene. It would appear that factors other than muscle damage contribute to IL-6 production such as energy crisis, metabolic, hormonal alterations and oxidative stress all known to stimulate systemic cytokine release (Cannon 2000).

In the current study, a post exercise increase in anti-inflammatory IL-4 was observed for both trials combined. The increase in IL-4 is likely to have been triggered by increased IL-6 production following the onset of exercise (Cox, 2007). Few studies have examined the acute plasma IL-4 response to exercise, and those that have often report no change. Niemen et al. (2001), reported post marathon levels of IL-4, IL-1 β , IL-2, INF- γ and IL-4 remained near pre-race or at non-detectable levels. Similarly, Suzuki et al. (2000) reported no change in plasma IL-4 concentration post a competitive marathon race. In agreement with the current study, Ostapiuk-Karolczuk et al. (2012) reported that IL-4 and IL-6 significantly increased immediately following exercise (90 minutes of running at 65% $\dot{V}O_{2\max}$). There are equivocal findings in previous literature. Differences may be due to exercise duration as Nieman et al. (2001) and Suzuki et al. (2000), both examined more prolonged marathon running.

According to Peake et al, (2005) when examining cytokine kinetics, IL-6 is the first cytokine to increase in the circulation during exercise. This is followed by proinflammatory IL-1 β and TNF α , followed by anti-inflammatory IL-4 and IL-10. Both the findings of Ostapiuk-Karolczuk et al. (2012) and the current study show a different pattern, with both IL-6 and IL-4 elevated immediately post exercise. In this study, no changes in pro inflammatory TNF α or IL1- β were detected. This finding is in contrast to previous work that has reported elevated TNF α and IL1- β following 1.5 and 2 hours of cycling (Cox et al., 2010; Brenner et al., 1999 respectively). In contrast, a number of studies have reported that systemic levels of TNF α and IL1 β

could not be detected, or only very small changes were measurable in plasma after eccentric exercise (Smith, 2000; Hirose, 2004). There are several possible explanations for variable findings in previous literature. Firstly, the type of exercise and its intensity and duration employed in the intervention protocol might affect the resulting cytokine profile. Physical exercise that induces muscle damage such as prolonged endurance exercise is associated with elevations in pro-inflammatory cytokines such as IL-1 and TNF α (Moldoveanu et al., 2000). It is possible the volume of the exercise employed in the current study, may not have been substantial enough to evoke a pro inflammatory cytokine response. In addition, plasma samples were only collected pre and immediately post exercise, therefore possible increases in proinflammatory cytokines later in the recovery period cannot be ruled out. For example, TNF α and IL-1 β have been found to gradually increase and peak 6 hours into the recovery period following 90 minutes running Ostapiuk-Karolczuk et al. (2012). Furthermore, Sprenger et al. (1992), suggested measurement of circulating levels of IL-1 β might not be an accurate measure of production, since assessment is complicated by rapid clearance from the circulation. IL-6 is also known to directly inhibit the expression of TNF α (Pedersen, 2000). In the current study, IL-6 and IL-4 were both elevated in the immediate post exercise period; there is the possibility this might have dampened a pro-inflammatory response.

In this study, there was a main effect of time for MCP-1. Specifically a post exercise increase in MCP-1 was observed in the time trial. This finding is in agreement with Peak (2005) who observed an elevation in MCP-1 concentration following 3 exercise protocols including, level treadmill running at 60% $\dot{V}O_{2\text{ max}}$, 1 hr treadmill running at 85% $\dot{V}O_{2\text{ max}}$ and downhill running at 60% $\dot{V}O_{2\text{ max}}$. In addition, Suzuki et al. (2003) reported increased concentration of plasma IL-4 and MCP-1 immediately after a marathon race. It was concluded the increase was most likely to mediate recruitment

and activation of neutrophils and monocytes to the site of muscle damage from the circulation. MCP-1 is a chemokine involved in mediating monocyte chemotaxis (Wells et al., 2016). Exercise related myotrauma activates myogenic precursor cells associated with training induced muscle growth (Bellamy et al., 2014). It has been suggested these cells depend on macrophages derived from monocytes to play a supportive role in their action (Seale et al., 2001). In contrast, Ihalainen et al. (2014) reported a significant decrease in plasma MCP-1 concentrations following an acute bout of leg press exercise (5 sets of 10 at 80% 1RM). Previous research on the MCP response to endurance and intermittent/ strength based exercise is limited and equivocal; however the differences in findings could be related to the differences between exercise protocols and their stimulus.

In this study, plasma VEGF concentration was higher pre-exercise for both trials combined. VEGF is an endothelial cell mitogen that stimulates angiogenesis by migration and endothelial cell proliferation (Hiscock 2015). It is important in vascular adaptation to exercise stress, depending on the metabolic and oxygen demands of the tissues (Gunga, 1999). Skeletal muscle cells/ endothelial cells produce VEGF, and a fraction can be secreted into the systemic circulation (Kraus et al., 2004). In humans, local VEGF mRNA expression has been reported to be elevated 1-hour post leg extension exercise (Richardson et al., 1999; Hiscock et al., 2015). Elevation in VEGF mRNA in skeletal muscle may increase VEGF protein synthesis, which is then released into the extracellular matrix and the circulation. Hiscock et al. (2015). Decreased serum VEGF concentration has been reported post Marathon running at high altitude (Gunga 1998). In contrast elevated plasma VEGF concentration has been reported immediately post 1 hour cycle ergometer exercise at 50% $\dot{V}O_{2\max}$ Kraus et al. (2004) and post marathon running at 2300m altitude (Schobersberger et al., 2000). Therefore, previous research findings regarding the plasma VEGF response to acute exercise are equivocal. Hiscock et al., (2015) observed a 9-fold

elevation in skeletal muscle VEGF mRNA post 3 hours of low intensity knee extension exercise at 50% peak workload. Femoral vein VEGF concentration increased post exercise however there was no change in femoral artery VEGF concentration. This suggests that VEGF may be released by the muscle and taken up by other tissues. If this is the case then the measurement of circulating VEGF might not necessarily reflect its increased release from skeletal muscle.

There are a number of confounding variables in the current study. Previous studies have measured pro and anti - inflammatory mediators at 6, 12, 24 and 72 h intervals post exercise (Brenner et al., 1999; Cox, 2007). Alternatively, some previous studies, similar to the present study, have measured cytokine responses in the acute pre and post exercise period. The time course of appearance of inflammatory and anti –inflammatory cytokines varies. For example IL-8 and TNF α peak in the hours and days post, exercise (Pedersen, 2000) therefore, the results of the current study) have limited precision and may not present the complete picture regarding immune changes following a bout of exercise.

Despite a high sensitivity kit being used in this study, some cytokines were at or below the minimum detection limit. This was particularly the case for IL-2, IL-10, IFNG, IL1 α and IL1- β . It should be taken into consideration that concentrations of cytokines in peripheral blood plasma may not reflect local up regulation or down regulation in the tissues. Therefore the inclusion of muscle biopsies/ urine samples and the measurement of arteriovenous differences across the exercising limb (and over more sampling time points), might assist us in understanding the cytokine response to exercise more clearly. In addition, the variability in analysis techniques employed in previous studies makes it difficult to make comparisons between study findings. Often different types of plate analysis are employed ranging from suspension array systems to custom manufactured multiple cytokine kits (Cox, 2010).

General day to day variability in plasma cytokine concentration should also be appreciated. In this study there was no difference in baseline concentrations of cytokines at rest prior to each of the two cycling trials however, a main effect of trial for IL-8 concentration ($p = 0.011$) was observed. Mean results show IL-8 concentrations were already elevated at rest prior to the time trial (7.12 ± 4.29) compared to prior the interval trial (4.29 ± 2.42). It is unknown as to why this difference in baseline values occurred. Trials were randomised so it is unlikely this was due to an order effect; it could possibly have been due to day-to-day variability in measurement or a sampling/ analysis error. Athletes' cytokine responses to exercise are not homogenous and they are influenced by many factors such as training status, age (Gokhale, 2006), stress hormones, oxidative stress and carbohydrate status (Cannon, 2000). Diet was not controlled to any formal extent in the current study, participants were asked to replicate their dietary intake for 24 hours and fast for 2 hours prior to laboratory visits. It has been established that feeding prior to an exercise test can affect hormone and cytokine levels (Venkatraman & Pendergast, 2002). Whether an athlete is illness prone may also play an important role in their immunological status at rest and during exercise. Cox et al, (2007). These factors should be considered when carrying out trials on different days or over a more prolonged testing periods.

The intensity for the cycling protocols employed in the current study, was set as a fraction of participants maximal aerobic power obtained in their initial $\dot{V}O_{2\text{ peak}}$ test. Therefore, if participants had not been motivated to reach their maximum power output during the initial test, they may have been working at a lower intensity than intended during the experimental trials.

Conclusion

In conclusion, the findings of the current study demonstrate that a 1 hour pre-loaded cycling time trial and a high intensity interval trial evoked an increase in IL-6 concentration post exercise. The magnitude of change was significantly higher in the 1-hour trial. This study provides evidence of an acute cytokine response post cycling exercise specifically regarding an elevation in IL-6, IL-4, MCP-1 and a decrease in VEGF post exercise compared to pre. This study attempted to provide a model of acute cytokine changes in response to cycling that represents the training sessions that cyclists might complete on a daily basis. The study contributes information to the field as the majority of previous research has examined extremely prolonged exercise, exercise known to induce muscle damage (Nieman et al., 2005; Gill et al., 2015) or prolonged exercise in hot environments (Cosico-Lima et al., 2011; Luk et al., 2016). Future studies should consider local and peripheral measurement of cytokine concentration at multiple time points post exercise in order to present the complete picture regarding immune changes following a bout of exercise.

A secondary aim of the present study was to investigate the relationship between saliva and plasma concentration of IL-1 β using the plasma values obtained from the main study and saliva values analysed using a separate saliva plate (Salimetrics UK). The relationship between Saliva and plasma IL-1 β values were examined pre time trial, post time trial, pre interval trial and post Interval trial (see table 3.3). A significant correlation between plasma and saliva measurements of IL-1 β was observed only in the post time trial data. This relationship between saliva and plasma was evident for both original saliva values and when saliva was expressed as concentration: Osmolality. When examining the change in IL-1 β values, pre to post exercise, for both cycling protocols (expressed as percentage change relative to 100), there was no relationship between plasma and saliva percentage change

in IL-1 β for the pre-loaded time trial. However, there was a relationship in percentage change for the interval trial; this was only evident when plasma was expressed as concentration: osmolality (table 3.4). Why a relationship between plasma and saliva assay measurements only existed in the conditions as stated, suggests a lack of consistency. Furthermore, Bland and Altman analysis indicated poor agreement between the assays when considering percentage change in IL-1 β for both exercise protocols (table 3.5 and figure 3.1)

The findings of this study are in agreement with previous work suggesting that the relationship between human IL-1 β levels in plasma and saliva is generally poor (Williamson et al, 2012). It is also possible that salivary samples are more reflective of local immune responses in the mouth or upper respiratory tract (Gaphor et al, 2014) rather than reflective of cytokine levels in the systemic circulation. It should be noted, in the current study, salivary flow rate was not measured. IL-1 β is not flow rate dependent with individuals whose IL-1 β levels are considered in the normal range. The effect of flow rate on individuals with elevated levels of IL-1 β is unknown and therefore it may have been relevant to measure salivary flow rate where the measured concentration is multiplied by the flow rate to result in product measured per unit of time. (Salametrics salivary IL-1 β ELISA kit manual) it is important to consider the location of samples used when attempting to quantify cytokine changes in response to exercise. More recently, research has considered a combination of local and peripheral measures including muscle biopsies to quantify myokine mRNA expression and muscle protein levels (Chan et al., 2004). In addition, plasma, serum and urine samples may provide further information on the concentration and clearance of cytokines in the systemic circulation (Nieman et al., 2001).

In conclusion, the relationship between saliva and plasma assays for the determination of IL-1 β was not consistent, there was not a good level of agreement

between the assays. Therefore, the measurement of IL-1 β values using saliva is not a valid replacement for plasma samples.

CHAPTER 4 VALIDATION OF THE USE OF A FINGERTIP
DRY BLOOD SPOT METHOD TO DETERMINE CELLULAR
n-3 AND *n*-6 PUFA INCORPORATION IN COMPARISON TO
VENOUS BLOOD ERYTHROCYTE ANALYSIS

INTRODUCTION

In recent decades, omega 3 long chain polyunsaturated fatty acid status (*n*-3 LC PUFA status), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been of interest to scientists. The EPA and DHA status of humans has been extensively investigated to determine the influence of these fatty acids on the prevention and treatment of chronic diseases such as asthma and exercise induced asthma (Mickleborough et al., 2006). There is a great deal of interest surrounding *n*-3 PUFA and its cardio protective effects, it has been reported to be therapeutic in the prevention of fatal arrhythmia (Leaf et al., 2008) and in the reduction of inflammatory cytokine production (Mickleborough et al., 2009).

In order for researchers to examine the relationships between dietary FA intake and biological outcomes, it is important that reliable biomarkers to measure FA status are available. Self-reported intakes from food frequency questionnaires (FFQ) and diet records can be used to estimate an individual's FA status, however these methods are reliant on memory, knowledge of portion sizes and participant motivation to complete data recording (Sun et al., 2007). Biomarkers are objective measures. There are a number of methods available to determine the fatty acid status of an individual however there is no universally accepted bio-marker to determine fatty acid status in response to increased dietary FA intake (Fekete, 2009).

The use of dried blood spots (DBS) is a convenient, rapid, and inexpensive method of sampling whole blood, which can then be analysed for PUFA content using traditional gas chromatography methods. DBS analysis was first employed by Marangoni et al. (2004), the method enables the analysis of fatty acid status using a capillary whole blood sample taken from the fingertip. Several variations of this

method have been developed by researchers since (Metherel et al., 2012; Harris & Polreis., 2016). However, current DBS blood sampling methods have some limitations due to possible contamination, and the stability of the samples is questionable due to oxidative losses of *n*-3 PUFA over time (Metherel, 2013).

Analysis of blood fractions including plasma or erythrocytes are commonly used as a surrogate measure of fatty acid tissue incorporation (Baylin & Campos, 2006). Serum *n*-3 PUFA fatty acid concentrations respond more quickly than erythrocytes to recent dietary intake due to a slower turnover rate of erythrocytes (Katan et al., 1997). The lifetime of an erythrocyte is approximately 120 days therefore erythrocyte phospholipid FA fractions are likely to be superior to plasma Fatty acid measurement and may provide a valid estimation of *n*-3 PUFA status that is more representative of long term intake (Katan et al., 1997).

Understanding accurately how dietary PUFA intake is reflected in erythrocyte phospholipids (or other blood fraction methods) allows us to validate indirect measures of self-reported intake, and to determine participant compliance with a dietary intervention or supplementation protocol. It may also allow us to determine relevant supplement dosage in studies that link disease states to dietary FA intake.

Conducting large epidemiological studies or intervention studies in the field (particularly in the case of athletic research) is limited by the need for a trained personnel to carry out venous blood collection from the antecubital vein, which in itself is invasive especially when multiple samples are necessary. In addition, the standard analysis of samples requires a number of time consuming steps.

Despite these limitations, venous blood erythrocyte percentage fractional content of (EPA) and (DHA) is still widely accepted as the most relevant biomarker in humans

to reflect long term *n*-3 PUFA status (Stark et al., 2016) and is considered gold standard by many investigators (Harris, & von Schacky, 2004; Poppitt et al, 2005). The aim of this study was to determine if alternate methods to venous blood sampling could provide a comparable measure of *n*-3 PUFA status whilst reducing some of the logistical limitations of venous blood sampling.

Specifically, the purpose of this study was to validate the use of a whole blood fingertip DBS method of blood collection and analysis to determine whole blood fatty acid composition and to compare this with the gold standard isolated erythrocyte analysis methods obtained via venepuncture. The following fatty acids were profiled using both methods (ALA), α -linolenic acid; (EPA), eicosapentaenoic acid; (DHA), docosahexaenoic acid; (total *n*-3 PUFA), total Omega 3; (LA), linoleic acid; (AA), arachidonic acid; (total *n*-6), total omega 6. (DHA + EPA), omega 3 index; (*n*-6/ *n*-3 PUFA), omega 6:3 ratio and (DHA/ EPA), docosahexaenoic acid: eicosapentaenoic acid ratio.

METHODS

Subjects

10 well trained healthy male competitive road cyclists and triathletes aged 42 ± 11 years (mean \pm SD) participated in the study. Participants were recruited via email from cycling and triathlon clubs in London and the South East and were a sub-group of the participants recruited for chapter 5. Well trained was defined as >6 hours of cycling per week for at least 12 months. Participants were excluded from the study if they had any incidence of illness or recent injury, evidence of hemodynamic or cardiovascular dysfunction, cigarette smoking, diabetes inflammatory or immune dysfunction and consumption of more than 2 fish containing meals per week. All participants reported they were not taking fish oil supplements, medication or had any allergies to fish/ soy or vitamin E related products. The study was approved by the University of Kent Research Advisory Group (REAG). The Participant's physical characteristics are presented in table 4.1.

Table 4.1. Participant physical characteristics (mean \pm SD)

Variables	Participants (n = 10)
Age (years)	$42. \pm 11$
Height (cm)	178.1 ± 7.5
Body mass (kg)	74.9 ± 9.5

Experimental design

Participants reported to the laboratory on three occasions over an eight-week period. All participants completed an informed consent form and a medical questionnaire. The first test was used to obtain baseline pre-supplementation blood samples. The second and third visits were used to obtain samples 4 weeks into and 8 weeks (post), the supplementation period. This is the first study to examine the

relationship between DBS and isolated erythrocyte values for fatty acid incorporation, over the duration of 8 weeks with a halfway sampling point. Participants were instructed to arrive fasted for 2 hours and in a rested, euhydrated state with water consumption permitted ad libitum.

Blood sampling procedures

Venous blood sample

An 8ml venous blood sample was collected by a trained phlebotomist using standard venepuncture procedure. Whole blood was collected directly into EDTA collection tubes from the antecubital vein. Red blood cells (RBC) were isolated from whole blood. The sample was transferred into a 50ml centrifuge tube with 8ml of Hanks balanced salt solution. This mixture was slowly layered on to 12ml of Ficoll-Paque premium and centrifuged at 400 x g for 35 minutes at 18° C. The concentrated RBC layer at the bottom of the tube was pipetted into a new 15ml tube and 3 x the volume of balanced salt solution was added and centrifuged at 400 x g for 10 minutes at a temperature between 18 – 20 ° C. RBC were then suspended in phosphate buffered saline (PBS) and aliquots were frozen at -80 °C.

Dry blood spot sample (DBS)

Prior to obtaining the DBS sample, Guthrie absorbent filter cards were pre-soaked in 5mg/ ml butylated hydroxytoluene (BHT) in methanol (Sigma-Aldrich) and allowed to air dry. This was in order to prevent/ limit fatty acid oxidation. The blood sample was collected from the fingertip using a lancing needle. Freely accumulated blood was then spotted onto four designated sample areas of the collection card and allowed to dry for several hours. Once dry, samples were sealed in zip lock bags.

All Samples were then freighted to Loughborough University and stored at -80°C. DBS samples were punched (30mm²) and frozen in Eppendorf's at -80°C.

Fatty acid analysis

A complete methodology for the procedures of fatty acid analysis can be found in appendix 1 including Total lipid extraction, lipid separation, transmethylation and gas chromatography/ mass spectrometry analysis.

STATISTICAL ANALYSIS

All statistical analysis was conducted using IBM SPSS version 24. A paired samples *t*-test was calculated to determine differences in FA percentage values for 10 different pairs of FAs measured by the DBS and venous method ($p = <0.05$). Simple linear regression was calculated to assess the linear relationship between capillary DBS FA values and the corresponding within subject fatty acids obtained from venous blood sampling. A two-way mixed measures (consistency) intra-class correlation was calculated to quantify the degree to which the two methods resembled each other. A Bland and Altman agreement test (Bland & Altman, 1986) was used to describe the limits of agreement between the methods. Linear regression was conducted to examine the relationship between the difference between the two methods and the mean of the two methods to correct for any proportional bias in the Bland and Altman analysis.

Analysis of pairs of data at 3 different time points (A) pre, B) 4 weeks and C) 8 weeks for each participant (30 samples in total)), violated the assumption of "independence of residuals" for linear regression (the samples are related or correlated). So the following samples were selected to examine if the DBS method

could be considered valid in comparison to venous erythrocyte methods across the entire supplementation period A, B and C

A = pre supplementation, B = mid supplementation and C = post supplementation

Participant	1	2	3	4	5	6	7	8	9	10
Sample time point	A	B	C	A	B	C	A	B	C	A

Figure 4.1 participant samples used for analysis

RESULTS

Analysis of difference in FA percentage values between venous erythrocyte analysis and whole blood DBS methods (Paired T-test)

There were 5 outliers in the data as assessed by inspection of a box plot for differences between paired values greater than 1.5 box lengths (table 4.2). The outliers were left in the data analysis as they were not considered extreme (not greater than 3 times the interquartile range from a quartile). Normal distribution was assessed by Shapiro-Wilk test ($p = > 0.05$). Fatty acid values were normally distributed.

Table 4.2 Outlier identification values and case number

Fatty acid	Case number	Venous value	DBS value	Difference
AA	1	19.97	4.16	15.81
	2	14.81	8.65	6.16
EPA	6	6.90	2.30	4.61
Total n-6	1	38.95	14.88	24.07
Total n-3	6	22.75	10.21	12.54

Values are presented as fatty acid % fraction.

The results of the paired samples *t*-test indicate the mean values for some of the FA differed significantly between methods. Specifically, the venous method, elicited statistically significantly higher values for EPA, DHA, AA, EPA + DHA & n-6/ n-3 PUFA than the DBS method (table 4.3). The DBS method produced a significantly higher value for ALA compared to the venous method. There was no difference between methods for DHA, LA, total n-3 PUFA or DHA/ EPA.

Relationship between DBS and venous methods

To assess linearity, a scatter plot of venous blood samples against DBS blood samples with superimposed regression line was plotted for each FA. Visual inspection of these plots indicated a linear relationship for EPA in particular. Residuals were normally distributed, as assessed by visual inspection of a normal probability plot.

Linear regressions to examine the relationship between capillary DBS and venous erythrocyte methods of measuring individual FA are summarised in table 4.4 and Figure 4.2. There was a significant correlation between methods of measurement for EPA ($R^2 = 0.64$, $p = 0.005$), a large effect size according to Cohen (1988). AA, Total n-6 and n-6/ n-3 PUFA correlated with a small non-significant effect size (AA, $R^2 = 0.25$, total n-6 PUFA $R^2 = 0.25$ and n-6/ n-3 PUFA, $R^2 = 0.29$). There was no association between DBS and venous methods for ALA, DHA, total n-3 PUFA, LA, EPA + DHA index or DHA/ EPA ratio.

Intra-class correlation coefficients showed the methods of measurement were similar for the determination of EPA status (ICC = 0.72) but not for AA (ICC = 0.49), total n-6 PUFA (ICC = 0.46), or n-6/ n-3 PUFA (ICC = 0.18). Cicchetti (1994) (see table 4.4).

Bland and Altman analysis showed moderate levels of agreement between methods for determining EPA % FA composition, mean difference = 1.12% with 95% limits of agreement -1.43% – 3.67% without any evidence of proportional bias $p = 0.05$. The remaining FA did not show good agreement between methods, in addition for the fatty acids , ALA, DHA, EPA + DHA and n-6/ n-3 PUFA (see table 4.5 and figure 4.2) there was evidence of proportional bias $p = <0.01$.

Table 4.3 Mean difference comparison of blood fatty acid percentage content derived from DBS and venous methods (mean \pm SD). Significant differences between methods was observed in ALA, AA, total n-6 PUFA, EPA + DHA & n-6/ n-3 PUFA ratio. No significant differences were observed in DHA, total n-3 PUFA, LA & DHA/ EPA.

Fatty acids	Venous Mean %	DBS Mean %	Paired mean \pm SD	95% CI Lower	95%CI upper	t	df	Sig (2 tailed)
(n-3) PUFA								
ALA	0.26 \pm 0.08	6.76 \pm 1.64	-6.51 \pm 1.64	-7.68	-5.36	-12.56	9	<0.001**
EPA	2.86 \pm 2.08	1.75 \pm 1.31	1.12 \pm 1.30	0.19	2.04	2.72	9	0.024*
DHA	5.11 \pm 3.66	2.79 \pm 0.86	2.33 \pm 3.74	-0.35	5.00	1.97	9	0.081
Total n-3 PUFA	12.41 \pm 4.94	12.71 \pm 2.43	-0.30 \pm 5.84	-4.48	3.87	-0.16	9	0.874
(n-6) PUFA								
LA	14.20 \pm 2.15	14.42 \pm 2.81	-0.22 \pm 3.30	-2.58	2.14	-0.21	9	0.841
AA	18.53 \pm 2.65	7.70 \pm 2.25	10.82 \pm 2.48	9.05	12.60	13.81	9	<0.001**
Total n-6	37.60 \pm 3.88	25.42 \pm 3.88	12.18 \pm 5.13	8.51	15.84	7.51	9	<0.001**
(Index & ratio)								
EPA + DHA	7.98 \pm 4.87	4.53 \pm 1.76	3.44 \pm 4.55	0.19	2.04	2.72	9	0.024*
n-6/ n-3 ratio	3.63 \pm 1.81	2.00 \pm 0.31	1.63 \pm 1.66	0.44	2.82	3.09	9	0.013*
DHA/ EPA	2.24 \pm 1.91	2.47 \pm 1.44	-0.23 \pm 2.10	-1.73	1.27	-0.35	9	0.736

Data are presented as individual fatty acid (% fraction mean \pm SD). ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n-3, Omega 3; LA, linoleic acid; AA, arachidonic acid; n-6, omega 6. Paired samples t-test *p < 0.05 **p < 0.01.

Table 4.4 The relationship between blood fatty acid percentage content derived from DBS and venous methods (mean \pm SD). There was a strong linear relationship between methods for EPA measurement (Cohen 1988) and a good intra-class agreement (Cicchetti 1994)

Fatty acids	N	Venous Mean %	DBS Mean %	R	R ²	ICC	F value	p-value	Regression equation
(n-3) PUFA									
ALA	10	0.26 \pm 0.08	6.76 \pm 1.64	0.053	0.003	0.005	0.023	0.884	Y= 0.24 + (0.002* X)
EPA	10	2.86 \pm 2.08	1.75 \pm 1.31	0.802	0.643	0.722	14.39	0.005**	Y= 0.63 + (1.28* X)
DHA	10	5.11 \pm 3.66	2.79 \pm 0.86	0.018	<0.001	0.008	0.002	0.962	Y= 4.90+ (0.08* X)
Total n-3 PUFA	10	12.41 \pm 4.94	12.71 \pm 2.43	0.156	0.024	-0.124	0.201	0.666	Y= 16.44 + (-3.17* X)
(n-6) PUFA									
LA	10	14.20 \pm 2.15	14.42 \pm 2.81	0.135	0.018	0.130	0.148	0.711	Y= 12.72 + (0.10* X)
AA	10	18.53 \pm 2.65	7.70 \pm 2.25	0.499	0.249	0.493	2.655	0.142	Y= 14.01 + (0.59* X)
Total n-6 PUFA	10	37.60 \pm 3.88	25.42 \pm 3.88	0.502	0.252	0.464	2.700	0.139	Y= 29.09 + (0.34* X)
(Index & ratio)									
(EPA + DHA)	10	7.98 \pm 4.87	4.53 \pm 1.76	0.358	0.128	0.229	1.173	0.310	Y= 3.35 + (0.99* X)
n-6/ n-3 ratio	10	3.63 \pm 1.81	2.00 \pm 0.31	0.573	0.288	0.178	3.239	0.110	Y= -2.69 + (3.15*X)
DHA/ EPA ratio	10	2.24 \pm 1.91	2.47 \pm 1.44	0.242	0.059	0.378	0.498	0.500	Y=0.64 + (0.67*X)

Data are presented as individual fatty acid (% fraction mean \pm SD). ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n-3 PUFA, Omega 3; LA, linoleic acid; AA, arachidonic acid;n-6 PUFA, omega 6. Simple linear regression *p < 0.05 **p < 0.01.

Table 4.5 Bland and Altman agreement between DBS and venous methods and simple linear regression of the mean of the two methods (X) and the difference between the two methods (X-Y) to determine the presence of systematic bias. There was good agreement between methods for EPA and n6/ n-3 PUFA

	(N)	Mean of V & DBS methods	difference mean (\bar{d})	$\bar{d}-1.96s$	$\bar{d}+1.96s$	R	R ²	F value	p-value	Regression equation
(n-3)PUFA										
ALA	10	3.51 ± 0.82	-6.51 ± 1.64	-9.72	-3.30	0.996	0.992	956.76	<0.001**	Y= 0.44 + (-1.98* X)
EPA	10	2.30 ± 1.61	1.12 ± 1.30	-1.43	3.67	0.629	0.396	5.251	0.051	Y= -0.05 + (0.51* X)
DHA	10	3.95 ± 1.89	2.33 ± 3.74	-5.00	9.66	0.896	0.803	32.668	<0.001**	Y= -4.69 + (1.78* X)
Total n-3 PUFA	10	12.56 ± 2.58	-0.30 ± 5.84	-11.74	11.14	0.614	0.377	4.843	0.059	Y= -17.79 + (1.39* X)
(n-6)PUFA										
LA	10	14.31 ± 1.88	-0.22 ± 3.30	-6.68	6.25	0.266	0.071	0.607	0.458	Y= 6.45 + (-0.47* X)
AA	10	13.11 ± 2.13	10.83 ± 2.48	5.97	15.68	0.184	0.034	0.282	0.610	Y= 8.00 + (0.22* X)
Total n-6 PUFA	10	31.51 ± 4.23	12.17 ± 5.13	2.13	22.22	0.433	0.188	1.850	0.211	Y= 28.72 + (-0.53* X)
Index/ ratio										
EPA+DHA	10	6.25 ± 2.87	3.45 ± 4.55	-5.47	12.36	0.789	0.623	13.211	0.007**	Y= -4.37 + (1.250* X)
n6/ n-3 PUFA	10	2.82 ± 1.00	1.63 ± 1.67	-1.64	4.90	0.958	0.918	89.331	<0.001**	Y= -290 + (1.61* X)
EPA/ DHA	10	2.36 ± 1.33	-0.23 ± 2.10	-4.34	3.88	0.281	0.079	0.688	0.431	Y= -1.28 + (0.44* X)

Data are presented as individual fatty acid (% fraction mean ± SD). ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n-3, Omega 3; LA, linoleic acid; AA, arachidonic acid; n-6, omega 6. Simple linear regression *p < 0.05, **p < 0.01.

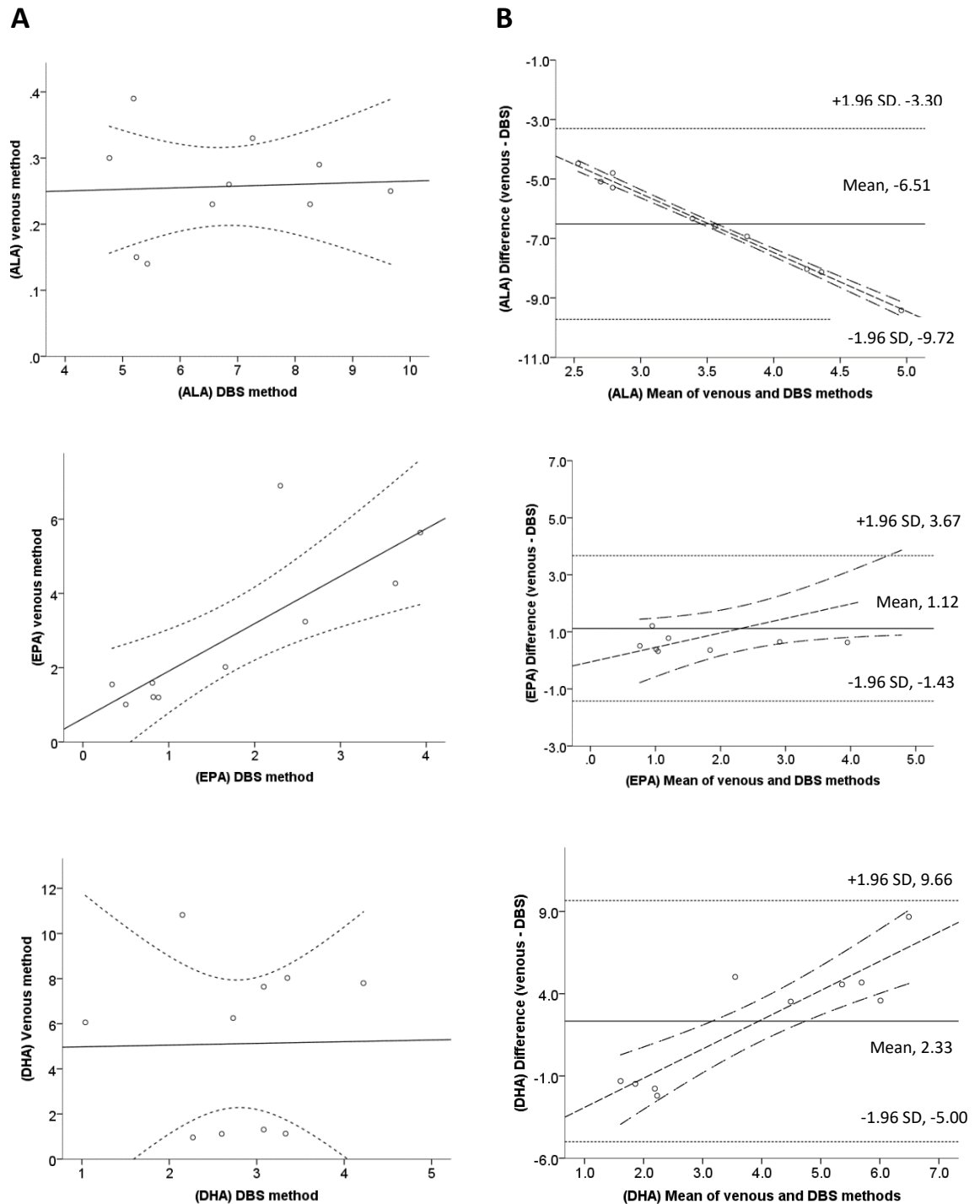


Figure 4.2 The relationship between venous and DBS derived measures of fatty acid percentage content. Each case is presented as a regression analysis (column A) and Bland and Altman plot where differences are presented as units (column B). Regression equation is expressed as $y = a$ (95% CI) + b (95% CI) x .

Regression relationship. $P = <0.05^*$, $P = <0.01^{**}$

In panel A, the solid line represents the line of identity and in panel (B) the dashed line represents the bias 95% limits of agreement. In panel (B) a regression line is the superimposed (dashed line) in order to determine any significant proportional bias.

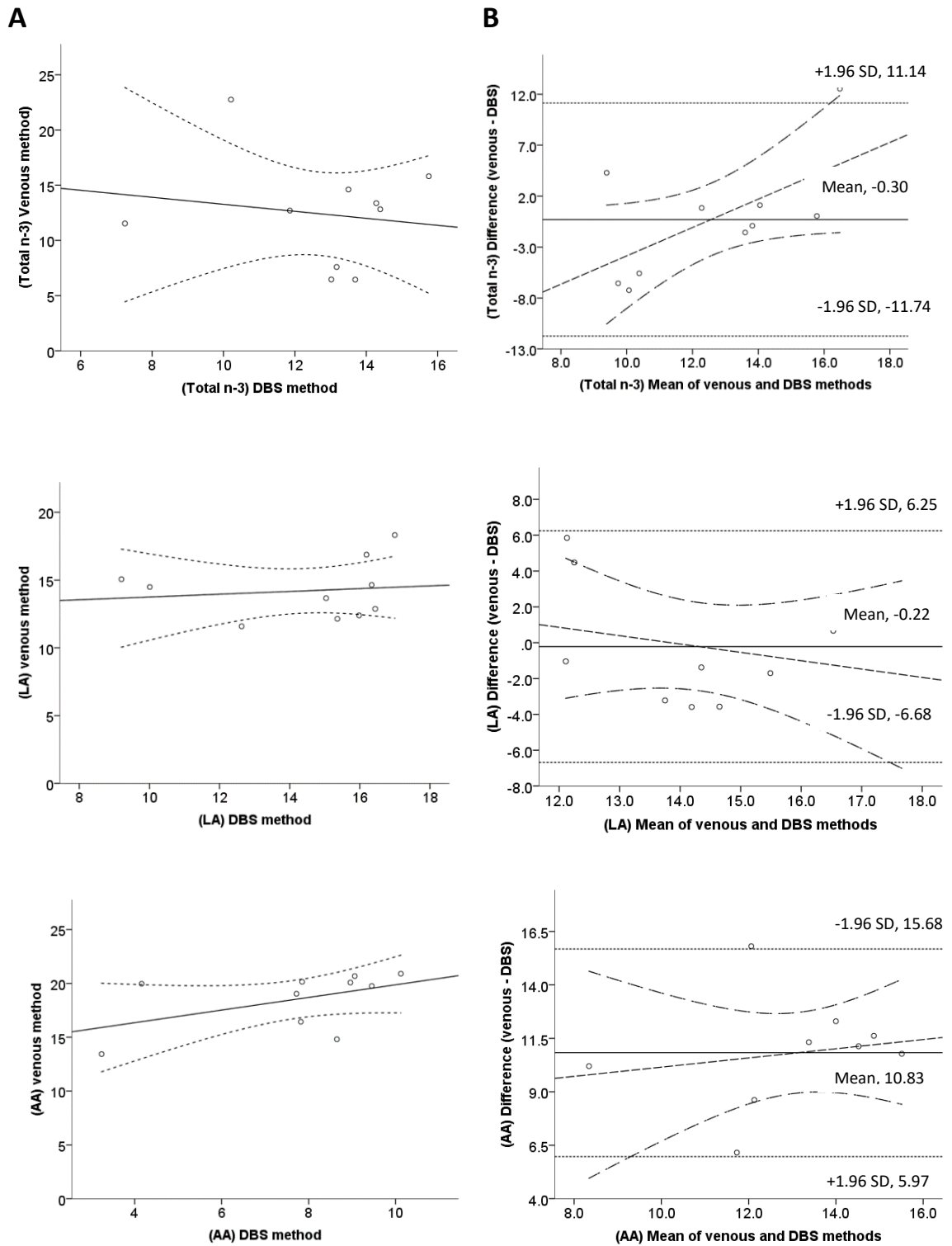


Figure 4.2 The relationship between venous and DBS derived measures of fatty acid percentage content. Each case is presented as a regression analysis (column A) and Bland and Altman plot where differences are presented as units (column B). Regression equation is expressed as $y = a + b x$.

Regression relationship. $P = <0.05^*$, $P = < 0.01^{**}$

In panel A, the solid line represents the line of identity and in panel (B) the dashed line represents the bias 95% limits of agreement. In panel (B) a regression line is the superimposed (dashed line) in order to determine any significant proportional bias.

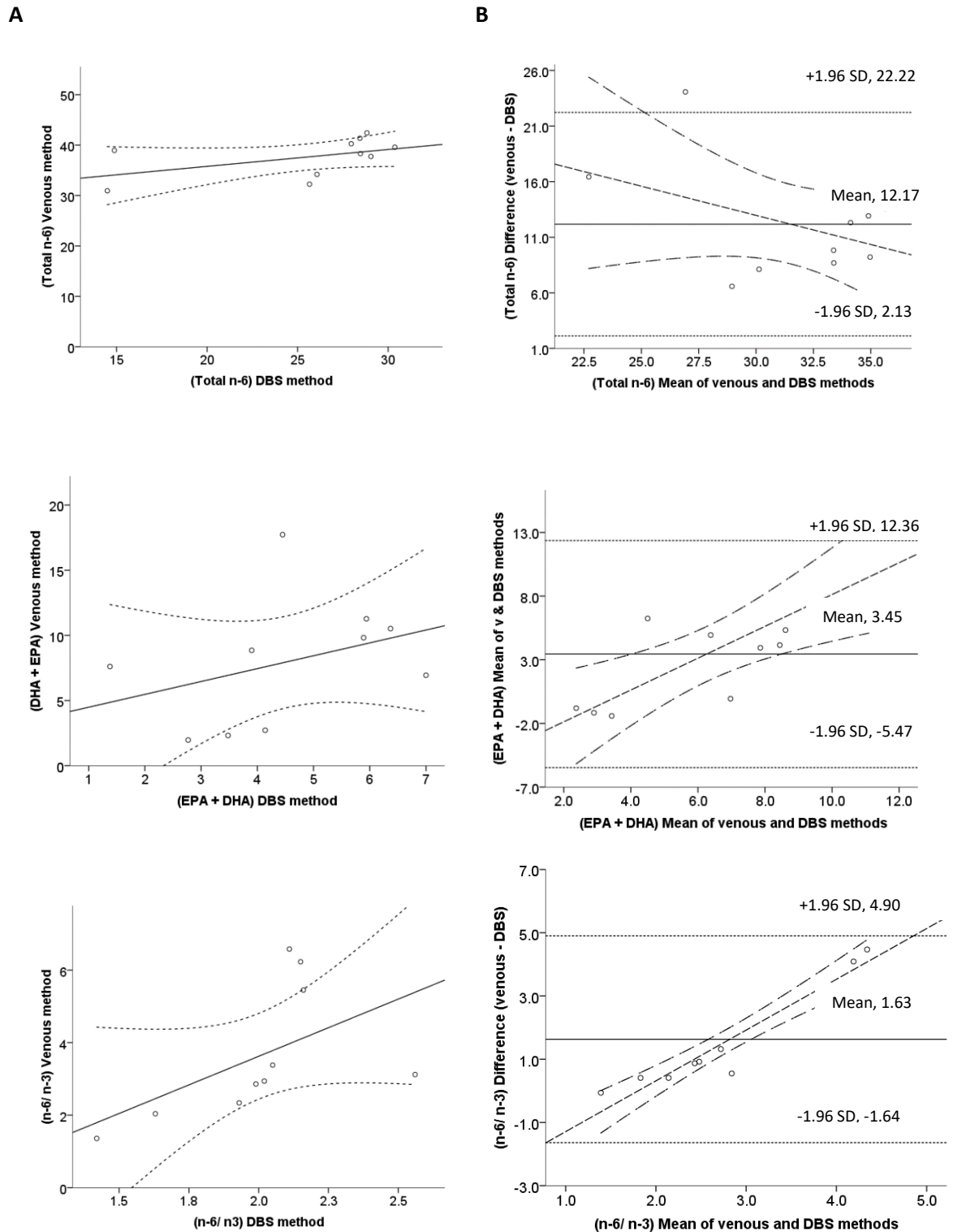


Figure 4.2 The relationship between venous and DBS derived measures of fatty acid percentage content. Each case is presented as a regression analysis (column A) and Bland and Altman plot where differences are presented as units (column B). Regression equation is expressed as $y = a$ (95% CI) + b (95% CI) x .

Regression relationship. $P = <0.05^*$, $P = <0.01^{**}$

In panel A, the solid line represents the line of identity and in panel (B) the dashed line represents the bias 95% limits of agreement. In panel (B) a regression line is the superimposed (dashed line) in order to determine any significant proportional bias.

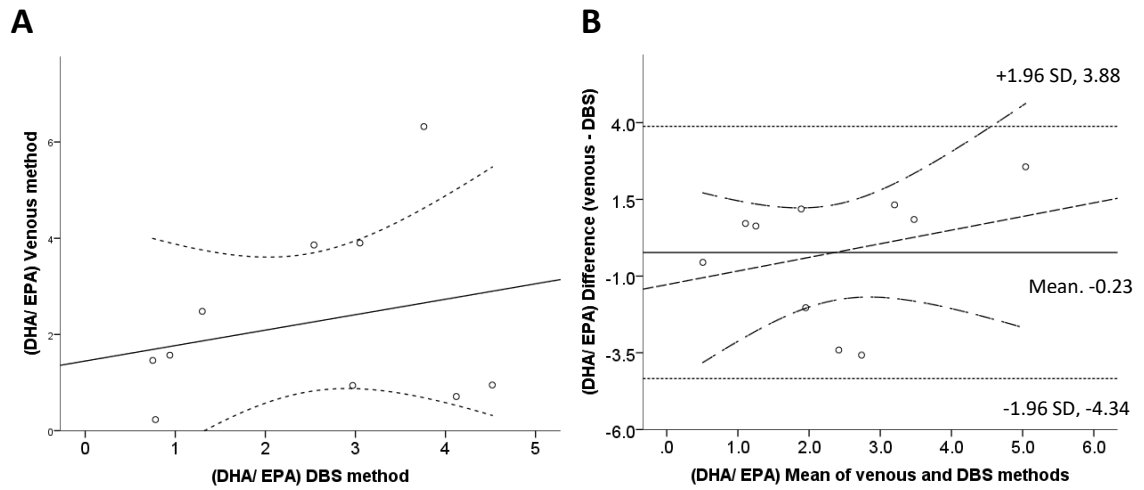


Figure 4.2 The relationship between venous and DBS derived measures of fatty acid percentage content. Each case is presented as a regression analysis (column A) and Bland and Altman plot where differences are presented as units (column B). Regression equation is expressed as $y = a$ (95% CI) + b (95% CI) x .

Regression relationship. $P = <0.05^*$, $P = < 0.01^{**}$

In panel A, the solid line represents the line of identity and in panel (B) the dashed line represents the bias 95% limits of agreement. In panel (B) a regression line is the superimposed (dashed line) in order to determine any significant proportional bias.

DISCUSSION

The aim of this study was to compare whole blood DBS methods analysis against conventional gold standard venous erythrocyte analysis for determining FA status.

Difference in FA values between the venous erythrocyte and capillary DBS methods of measurement

When the values for each FA were compared between the two methods of measurement (DBS and isolated erythrocytes), a significantly higher mean percentage of EPA, AA, total n-6 PUFA, EPA+DHA and n-6/ n-3 PUFA values were observed in the isolated erythrocyte method in comparison to DBS. In contrast, ALA values were significantly lower in isolated erythrocytes compared to DBS. No differences were found between methods for DHA, total n-3 PUFA, LA, and DHA/ EPA ratio (table 4.3).

The difference in values observed between methods were expected due to the higher RBC cell count in the isolated erythrocyte method of analysis compared to the DBS whole blood capillary sample. Whole blood samples contain plasma and a number of other cell types including monocytes. It has been reported in previous research that PBMC's readily incorporate EPA and DHA into membrane phospholipids when supplementing with *n*-3 PUFA (Metherel et al., 2009). However, the levels of *n*-3 PUFA fatty acids in PMBCs are lower, as RBCs have a slower turnover with a longer lifetime of approximately 120 days in the circulation. Furthermore, their FA concentration is not affected acutely by recent dietary changes as would be the case with the serum fraction of the whole blood capillary sample (Katan et al, 1997). This is further confirmed by observations in previous studies that show good agreement between whole blood capillary DBS and whole

blood venous FA values (Min et al., 2011; Armstrong et al., 2008). Therefore, if capillary and venous whole blood samples had been compared in the current study the mean values for each FA may have been similar. It would have been interesting if we had dropped some of the whole venous blood sample (prior to erythrocyte isolation) onto a Guthrie card. This would have allowed us to determine if it was specifically the blood components that caused the differences between methods or the sample analytical procedure. In the current study there was no significant difference observed between methods for concentrations of DHA although levels were generally higher in the venous sample. The mean percentage difference between methods of measurement for EPA, DHA, and AA values in the current study are similar to the values reported in previous studies (Bailey-Hall et al., 2008 and Bell et al., 2011). See difference column entitled difference (DIFF) in table 4.6 below.

Table 4.6 Mean percentage blood sample fraction fatty acid content and the magnitude of difference between venous erythrocyte and DBS values in the current study compared to previous studies.

FA	Current study			(Bell et al, 2011)			(Bailey-Hall et al, 2008)		
	VEN	DBS	DIFF	VEN	DBS	DIFF	VEN	DBS	DIFF
EPA	2.86	1.75	0.78	1.49	1.13	0.36	0.76	0.78	-0.02
DHA	5.11	2.79	2.32	4.84	1.11	3.73	4.89	2.76	2.13
ALA	0.26	6.76	-6.5	0.15	0.04	0.11	0.320	0.55	-0.23
Total n-3	12.41	12.71	-0.3	9.21	2.08	7.13			
LA	14.20	14.42	-0.22	9.69	1.33	8.36	14.59	23.66	-9.07
AA	18.53	7.70	10.83	13.01	1.61	11.4	12.24	8.06	4.18
Total n-6	37.60	25.42	12.18	27.05	2.61	24.44			

Values are presented as fatty acid mean % fraction.

Due to the current study employing the sample procedure in figure 4.1, the absolute mean values in table 4.6 cannot be compared. We employed a systematic sampling method over the course of an 8-wk supplementation period, whereas the values

from Bell et al., (2011) and (Bailey-Hall et al., 2008) were baseline samples taken from participants attending a health clinic without supplementation. The difference between the values for the venous and DBS method can be compared, assuming there is not any systematic bias in either method in its measurement across a range of values.

Relationship and agreement between venous erythrocyte and capillary DBS methods for the determination of FA concentration

In the present study the relationship between erythrocyte derived FA values and DBS values for EPA correlated well ($R = 0.80$, $R^2 = 0.64$, $p = 0.005$), showing a large effect size according to Cohen (1988) (table 4.4). This finding is consistent with Bailey-Hall et al. (2008), who reported $R^2 = 0.68$ for EPA. There was no association between DBS and venous methods for ALA, DHA, total $n-3$ PUFA, LA, EPA + DHA Index or DHA/ EPA ratio. In contrast, Bell et al, (2011) reported a good correlation between methods for total $n-3$ PUFA $R^2 = 0.95$. Furthermore, Bell et al., (2011) and Bailey-Hall et al (2008) found good correlation between methods for DHA $R^2 = 0.58$ and 0.74 respectively. The reasons for the difference in findings is unclear. The study samples in Bell et al., (2011) were the same as the current study (whole blood capillary samples compared against venous erythrocyte samples). However, in Bell et al., (2011), resting blood samples were taken from healthy participants who were not supplementing with fish oil. Base line samples were compared rather than systematically selected samples from three time points of supplementation over 8 weeks (as in the current study). Therefore, it is possible that supplementation with $n-3$ PUFA may have changed the general pattern of FA incorporation in a dose response manner over the 8 weeks, possibly as a result of systematic bias which affected the relationship between the two sample types in the current study.

Intra-class correlation coefficients confirmed excellent similarity between methods for EPA measurement (ICC = 0.72) but not for AA (ICC = 0.49) or total n-6 PUFA (ICC = 0.46) (Cicchetti 1994). All other fatty acids had poor agreement between the two methods. (Table 4.4)

Bland and Altman analysis showed moderate agreement between methods for determining EPA% FA composition, mean difference = 1.12% with 95% limits of agreement -1.43% – 3.67% without any evidence of proportional bias $p = 0.05$. The remaining FA did not show good agreement between methods. In addition for the fatty acids, ALA, DHA, EPA + DHA and n-6/ n-3 PUFA, not only was there poor agreement, but there was also evidence of proportional bias $p < 0.01$ which suggests that the FA values derived from the two methods do vary through the range of measurement (table 4.5 and figure 4.2).

Some of the variances in measurement values in the current study compared to previous studies may be due to inconsistency of analysis steps. Ohta et al. (1990) described a method of DBS collection which involved initial separation of whole blood into PL and RBC. Thin layer chromatography (TLC) was carried out prior to fatty acid methyl ester (FAME) analysis to extract the fatty acids from phospholipids. Marangoni et al. (2004), described a whole blood fingertip sampling method that was applied directly to chromatography paper similar to the present study with the exception that the samples were stored at -80°C rather than at room temperature. Studies also vary in their methods of transesterification (Armstrong et al., 2008).

Furthermore, differences in the venous and DBS values in the current study may be due to a number of factors. For example, the degradation of samples due to oxidation is a known limitation regarding DBS sampling. Bailey-Hall et al. (2008)

collected DBS on BHT treated filter paper in order to minimise fatty acid oxidation (as in the current study). However Bell et al. (2011), carried out a blood stability trial and found no significant difference with the use of blood collection paper pre-treated with BHT compared to non-treated paper for analysis of AA, EPA and DHA. DHA percentage values reduced over a 4-wk storage period at room temperature from 2.93% to 1.95% (with most degradation occurring after 48 hours). The other fatty acid values reduced in a similar way. This suggests BHT may not necessarily improve sample stability.

In conclusion, the finger stick whole blood DBS method is a rapid non-invasive and practical field method to determine EPA status in individuals. However, this is not the case for other FA. The DBS method is not a replacement method for isolated erythrocyte analysis. In addition, due to inconsistencies in methods across studies and the concern regarding sample stability and contamination, the use of this method should be treated with caution. In relatively small intervention studies, where tissue fatty acid status is important for determining the success of a dietary intervention or supplementation protocol on tissue levels of *n*-3 and *n*-6 PUFA. It is likely that venous erythrocyte determination of the fatty acid profile is the most valid method. Consequently, for study 3, isolated erythrocytes were analysed from venous blood collection in order to determine the fatty acid status of cyclists.

CHAPTER 5 THE EFFECT OF A HIGH *n*-3 PUFA AND LOW
n-6 PUFA DIETARY INTERVENTION ON ERYTHROCYTE
N-3 PUFA PHOSPHOLIPID INCORPORATION, AND ITS
EFFECT ON SUBMAXIMAL CYCLING AT FOUR
DIFFERENT WORK RATES AND MAXIMAL AEROBIC
FITNESS ($\dot{V}O_{2\text{ peak}}$)

INTRODUCTION

Increased consumption of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may influence a number of physiological parameters related to exercise, cardiovascular health and circulatory function. Animal studies have shown that *n*-3 PUFA incorporation into myocardial cell membranes enhances the mechanical performance of the heart (McLennan et al., 1989), reduces the risk of arrhythmia in marmoset monkeys (McLennan et al., 1992), and reduces fatal arrhythmia (Burr et al., 1989). Furthermore, fish oil feeding has shown to reduce myocardial oxygen consumption by improving the energy efficiency of heart muscle, and lowering resting heart rate (HR) and blood pressure (BP) in healthy human participants (Shah et al., 2007).

Within the context of exercise training and performance, there is emerging evidence that *n*-3 PUFA supplementation has the potential to modulate a number of cardiovascular responses to exercise that could theoretically enhance endurance performance or adaptation to exercise. To date, human studies that have examined the influence of *n*-3 PUFA on performance have been conflicting. Zebrowska et al. (2014), observed a reduction in exercising HR, HR_{max} and increased $\dot{V}O_{2\max}$ in elite cyclists completing incremental exercise to exhaustion following *n*-3 PUFA supplementation. Cyclists supplemented with 1.3 g/ day of *n*-3 PUFA delivering 660 mg EPA and 440 mg DHA for 3 weeks. In addition, significant differences between pre and post intervention NO levels were observed following *n*-3 PUFA supplementation compared with the placebo group. NO increase was associated with enhanced endothelial function (measured by flow-mediated dilation (FMD)). It was suggested this mechanism augmented blood flow to the exercising skeletal muscle and may have improved $\dot{V}O_{2\max}$ performance. *N*-3 PUFA supplementation has been shown to enhance endothelial function, local

vasodilation and blood flow in skeletal muscle during rhythmic handgrip contractions (Walser & Stebbins, 2008). There are a number of direct and indirect mechanisms that may explain the effect of *n*-3 PUFA on NO production. Three isoforms of NO synthase (NOS) are involved in the production of NO. NOS I, located in neuronal cells (central and peripheral). NOS II, induced by macrophages and a variety of other cell types, and NOS III, an isoform expressed in endothelial cells. These isoforms use L-arginine as a substrate. NOS III is most influential in the vasculature, NOS I and II exert effects on the cardiovascular system (Li & Forstermann, 2000). EPA increases the synthesis of NOS III derived NO in the vascular endothelium; this improves endothelium-dependent vasodilation by increasing intracellular Ca^{2+} concentration (Wu, Cynthia & Meininger, 2002). This primes NO synthesis and induces the translocation of endothelial NOS (Park et al., 1996). Furthermore, *n*-3 PUFAs reduce oxidative stress. Elevated levels of reactive oxygen species (ROS) can damage the integrity of vascular endothelium and can contribute to atherosclerosis. *n*-3 PUFAs also modulate inflammation and produce resolvins and protectins that may contribute to decreased leukocyte-endothelial interactions (Casos et al., 2010). Some of these indirect actions might contribute to the therapeutic action of *n*-3 PUFA on the cardiovascular system. *n*-3 PUFA has also been associated with reduced blood viscosity (Oostenbrug, 1997). Blood viscosity is an important factor influencing maximal cardiac output and peripheral blood flow, both of which are important determinants of maximal aerobic capacity (Joyner & Coyle 2008).

Dietary fish oil may have the potential to modulate cardiovascular function during exercise due to the collective mechanisms discussed however; previous authors have yet to substantiate an improvement in endurance performance. Leaf and Rauch (1988) demonstrated that *n*-3 fatty acid supplementation increased maximal aerobic power ($\dot{V}\text{O}_{2\text{ max}}$). This experiment has been criticized as the researchers

used a constant treadmill speed with gradient increments every 3 minutes until exhaustion, which may have affected participant motivation and accuracy of $\dot{V}O_{2\max}$ measurement (Raastad et al, 1997). In addition, the observations by Leaf and Rauch have not been supported by subsequent studies. $\dot{V}O_{2\max}$ before and after *n*-3 PUFA treatment was unchanged after *n*-3 PUFA was supplemented with or without training (Brilla & Landerholm, 1990; Warner et al., 1989). Oostenbrug et al., (1997), found 3 weeks of dietary *n*-3 PUFA supplementation reduced red blood cell deformability by 2% during exercise however; there was no effect on time to complete a fixed workload in well-trained male cyclists. 10 weeks of *n*-3 PUFA supplementation did not improve maximal aerobic power, or running performance in trained football players (Raastad et al., 1997). Peoples et al. (2008), found no change in $\dot{V}O_{2\max}$ in endurance trained male cyclists following *n*-3 PUFA supplementation, however whole body O_2 consumption was reduced during submaximal steady state cycling. Five weeks of supplementation with *n*-3 PUFA, reduced BP (diastolic) and HR during submaximal exercise, however there were no observed improvements in time trial running performance (Buckley et al, 2009). In trained cyclists, relative oxygen consumption was significantly reduced in the *n*-3 PUFA supplemented group during a 5 minute maximal cycling time trial, however there was no difference in time trial performance (Hingley et al, 2017). In order to further examine the effect of *n*-3 PUFA on the $\dot{V}O_2$ cost of submaximal exercise and maximal aerobic capacity we examined the following three concepts.

1. *O₂ cost of submaximal exercise*

O₂ cost is related to cycling economy and is a significant component of endurance performance (Abbiss & Laursen, 2005). Studies that have examined the effect of *n*-3 PUFA supplementation on the oxygen cost of exercise are equivocal largely due to the number of different exercise modes/ intensities and durations being employed in previous work. The O₂ cost of exercise has been examined mainly during time trial protocols (Buckley et al., 2009; Hingley et al., 2017), time to exhaustion tests (Raastad et al, 1997) and during single bouts of fixed intensity exercise (Borlotti et al., 2007; Peoples et al., 2008). Kawabata et al. (2014) used a fixed rate submaximal protocol at workloads equivalent to 2 and 3mmol of blood lactate. To our knowledge, no studies to date have examined the influence of *n*-3 PUFA supplementation on oxygen consumption during exercise at four different submaximal workloads similar to the gross efficiency protocol used by Passfield & Doust (2000). Therefore, one of the primary aims of this study was to examine the effect of *n*-3 PUFA supplementation on whole body oxygen cost ($\dot{V}O_2$), HR, BP and mean arterial pressure (MAP) at four submaximal workloads, 100 W, 125 W, 150 W, and 175 W. In previous studies, the oxygen cost of exercise muscle has generally been inferred by pulmonary oxygen uptake kinetics. Near infrared spectroscopy (NIRS) can be used as a non-invasive method to monitor muscle oxygenation based on the principle that the NIR light absorption characteristics of haemoglobin (Hb) and myoglobin (Mb) are dependent on oxygen saturation. NIR light absorption changes in muscle reflect venous, arterial, capillary and intramuscular oxygenation (Van Beekvelt, 2001; Grassi, 2003; Shibuya, 2003). Therefore, a further aim of this study was to ascertain local tissue oxygenation (TSI%) and oxygen extraction (deoxyhaemoglobin) at the site of tissue under consideration (Vastus lateralis), using near-infrared spectroscopy (NIRS) in addition to measurement of pulmonary whole body $\dot{V}O_2$.

2. Maximal aerobic performance

Theoretically, *n*-3 PUFA fatty acid supplementation may enhance the delivery of oxygen when an individual is placed under conditions of increased O₂ demand, and may improve maximal aerobic performance (Zebrowska et al, 2014; Walser & Stebbins, 2008). Therefore, this study aimed to examine the effect of *n*-3 PUFA supplementation on maximal aerobic performance $\dot{V}O_{2\text{ peak}}$, Power_{max}, HR_{max} and HR relative to $\dot{V}O_{2\text{ max}}$ $\dot{V}O_2 / \text{HR}_{\text{max}}$.

3. Integrated dietary intervention

Previous studies that have examined the effect of *n*-3 PUFA supplementation on physiological parameters and exercise performance are lacking control regarding the background diet of participants. Over the past century the human diet has become enriched with the intake of *n*-6 PUFA linoleic acid (LA, 18:2 *n*-6) with a reduction in intake of alpha linolenic acid (ALA, 18:3 *n*-3 PUFA). Higher dietary LA intake has led to a change in the ratio of LA: ALA in the human diet from our evolutionary intake (observed in animals) of 2:1 to approximately 15:1 (Simpoloulos 1991). The Animal and human studies already discussed have demonstrated dietary *n*-6 and *n*-3 PUFA can alter cellular metabolism and mechanisms. EPA also competes with the same desaturase enzyme in the cyclo-oxygenase pathway as AA resulting in the production of less inflammatory mediators (Calder 2007). Enriched dietary intakes of LA may interfere with the metabolism of ALA into EPA. Therefore, the lowering of *n*-6 PUFA could possibly augment the benefits of parallel *n*-3 PUFA fatty acid supplementation. Ghosh et al, (2007) fed young male pigs with one of three diets that differed in *n*-6: *n*-3 PUFA ratio: 1) low LA & ALA b) Low LA & High ALA c) High LA & High ALA. The cardiovascular benefits of ALA were only observed when LA was reduced concurrently. As far as we are aware, few studies

have examined a dietary manipulation to lower *n*-6 PUFA intake in humans. MacIntosh et al. (2013), examined an average *n*-3, low *n*-6 PUFA diet (L6) and a high *n*-3, low *n*-6 PUFA diet (H3-L6) on EPA, DHA incorporation into erythrocytes. It was found that both the L6 and H3-L6 diets reduced erythrocyte *n*-6 PUFA content but L6 did not influence AA content. EPA and DHA increased in both L6 and H3-L6 however, H3-L6 increased EPA and DHA significantly more than L6, and reduced AA. The changes were attributed to increased consumption and therefore increased competition of *n*-3 EPA and DHA for metabolism. It is possible that a low *n*-6 PUFA dietary intake will augment EPA and DHA incorporation and lower AA desaturation. Therefore, a further aim of this study was to create an 8-week integrated dietary intervention designed to achieve reductions in background dietary *n*-6 PUFA whilst increasing *n*-3 PUFA (with supplementation) in the fish oil (FO) treatment group. In order to maintain a double blind design, a placebo dietary intervention that did not change *n*-6 PUFA dietary fatty acid intake was designed for the placebo group.

It was hypothesized that whole body O₂ cost ($\dot{V}O_2$) and tissue oxygen extraction (deoxy-HHb), HR and BP would be lower in the high *n*-3 PUFA/ low *n*-6 PUFA group at each submaximal cycling workload in comparison to the placebo group. In addition, it was hypothesized that FO would increase tissue saturation index (TSI %), $\dot{V}O_{2\text{ peak}}$, power_{max}, and decrease HR relative to $\dot{V}O_{2\text{ max}}$ ($\dot{V}O_{2\text{ max}}/HR_{\text{max}}$) post supplementation in comparison to the placebo group.

METHODS

Participants

18 well trained healthy male competitive road cyclists/ triathletes (well-trained was defined as having been involved in cycling training ≥ 6 hours per week for the past 12 months) aged 44.3 ± 9.5 years participated in the study. 3 participants did not

complete the study due to lack of time availability. The exclusion criteria used in order to eliminate factors that may have affected cardiovascular parameters were as follows: evidence of hemodynamic or cardiovascular dysfunction, cigarette smoking, diabetes or impaired regulation of glucose, inflammatory or immune dysfunction and consumption of more than 2 fish containing meals per week. All participants reported they were not taking fish oil supplements, or had any allergies to fish/ soy or vitamin E related products. The participants were competitive road cyclists or triathletes competing at club level ($n = 10$) or cyclists training regularly for the past 12 months (≥ 6 hours of cycling per week) ($n = 6$). The well-trained status of this group ensured less variation in muscular fatigue during peak O_2 consumption testing, (Bassett & Hawley 2000). Data collection was carried out over a 6-month period. Participant data for age, height, body mass, $\dot{\text{V}}\text{O}_{2\text{ peak}}$, resting heart rate (HR), resting blood pressure (BP) and sum of 7 skinfolds (Jackson and Pollock, 1978) are presented in table 5.3. The study was approved by the University of Kent Research Ethics Advisory Group (REAG).

Experimental design

Participants were randomly allocated in a counterbalanced double blind manner to two independent groups. Group 1 (FO $n = 7$) completed 8-weeks supplementation of $n-3$ PUFA combined with a dietary manipulation to lower background $n-6$ PUFA. Group 2 (SO $n = 8$) completed 8 weeks' supplementation with organic soy oil (placebo) combined with a placebo dietary manipulation designed to maintain the participant's habitual $n-6$ PUFA intake, treatments were double blind. Participants visited the laboratory on four occasions, with a minimum of 7-days rest between visit 1, familiarity trial and visit 2, pre-supplementation experimental trial. The 8-week supplementation and dietary manipulation treatment began on the day following visit 2. Visit 3 occurred 4 weeks into the supplementation period (halfway point blood

sampling), Visit 4, (post-supplementation experimental trial), occurred at the end of the 8-week supplementation period. On visits 2, 3 and 4 resting venous blood samples were collected in order to quantify the participant's' *n*-3 PUFA and *n*-6 PUFA status. This was to establish if there was a dose response relationship in tissue incorporation of *n*-3 PUFA over time. In addition, a dry blood spot (DBS) sample was collected at the same time in order to examine the validity of DBS sampling in comparison to the traditional venous RBC erythrocyte method (see chapter 4 and appendix 1 for more detail on blood sampling methods and analysis).

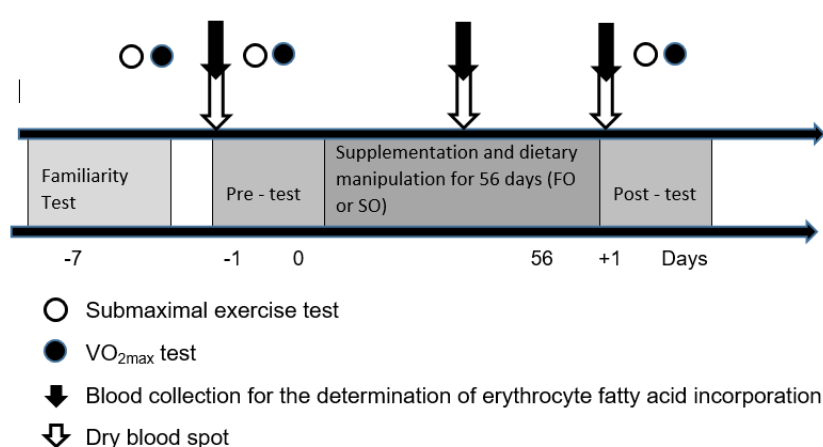


Figure 5.1. A schematic diagram illustrating the experimental protocol

Participants reported to the laboratory in a euhydrated and 2 hour fasted state, having abstained from caffeine, alcohol and exercise for 24 hours. Participants attended each visit at the laboratory at a similar time of day.

Visit 1

All Participants completed an informed consent form and a medical questionnaire. Participants then completed a familiarity trial according to the procedures detailed in experimental trials 2 and 4 (pre/ post supplementation). In addition, participants were instructed how to complete a 7-day diet record to be submitted via email to the experimenter the day prior to visit 2. A 15-minute discussion covered details

regarding how to record dietary intake using weights, household measures and portion sizes. A detailed example of what a complete food record should look like was also provided in accordance with recommendations for improving food record accuracy (Kolar et al., 2005). In addition, participants were asked to provide a list of other commonly consumed meals and snacks that may not have been included in the 7-day recording period.

Visits 2 and 4

Pre supplementation and post supplementation experimental procedures

Participants adjusted the Lode cycle ergometer (Excalibur sport version 2 Lode BV Groningen, Netherlands); all bike settings were recorded and repeated in the post supplementation test. Following a 10 min seated rest, participant's age, height, body mass, resting heart rate (Polar RS400 watch and wear link) and resting blood pressure (Tango + stress BP SunTech Medical. Inc. US) were recorded.

Pre-test blood samples were then collected: A 7ml blood sample was taken from the participant's antecubital vein in the forearm in a heparinized EDTA vacutainer for the determination of erythrocyte fatty acid status. 2 x 10µl fingertip capillary samples were collected from participants using an accucheck needle for the analysis of resting whole blood lactate and blood glucose levels (Biosin C-Line EKF Diagnostics). The second 10µl finger prick blood sample was applied to a Guthrie absorbent filter card to determine the validity of this method compared to traditional venous isolated erythrocyte methods of analysis (see chapter 4).

Muscle oxygenation was assessed using a Near Infrared Spectrophotometer (NIRS) (Oxymon Mk III Artinis Medical Systems BV, Netherlands). NIRS is a continuous

non-invasive optical method that emits continuous wavelengths of 780 and 850-nm on the vastus lateralis for the measurement of relative concentration changes in tissue oxygenated haemoglobin (O_2Hb), and deoxygenated haemoglobin (HHb) during exercise (Ryan et al, 2012). The NIRS derived HHb signal measures changes in muscle microvascular deoxygenation reflecting the relationship between local muscle O_2 delivery and muscle O_2 utilization at the site of optode placement (DeLorey et al, 2003). By definition, $[O_2Hb]$ and $[HHb]$ should exist in equilibrium, an increase in O_2Hb and a decrease in HHb is evidence of improved oxygenation, this would be the case if regional blood volume remained constant. However, measurement of O_2Hb is complicated during exercise due to its reflection of regional arterial blood flow (which is mostly oxygenated), whereas the O_2Hb signal reflects changes in O_2 extraction therefore is unaffected by changes in arterial blood flow (Grassi et al, 2003; Kowalchuk et al, 2002) Therefore HHb and TSI% were used for analysis.

The NIRS optode was placed 10cm above the lateral epicondyle on the participant's vastus lateralis with transmitters-receptor probes interspaced by 4.0 cm. The optode was taped into position and covered with a bandage to prevent contamination from ambient light; the sampling frequency was set to 10 Hz. A baseline measure of resting muscle oxygenation was taken for 2 minutes prior to starting the 4-stage submaximal exercise test, data was recorded continuously throughout. Mean minute values were analysed (taken from the final minute of the baseline measure and from the 4th to 5th minute of each submaximal exercise bout at 100 W, 125 W, 150 W, and 175 W).

4 stage submaximal cycling test

Following a standardized warm up at 100 watts for 10 minutes, participants completed a series of 4 x 7-minute sub-maximal cycling bouts at 100 W, 125 W, 150 W, 175 W, at a self-selected cadence on a cycle ergometer. Average cadence for each work rate was recorded and participants were asked to repeat the same cadence in their post-test (visit 4). Participants rested passively for 5 minutes between each stage. For each of the 4 exercise bouts, HR and expired gases were collected during minutes 4-5 in a non-diffusible mylar Douglas bag (Hans Rudolph Inc., KS, USA) and analysed (Servomex 5200, Servomex, Crowborough, UK) according to the procedures of Hopker et al, (2012). Rating of perceived exertion (RPE) (Borg, 1970) was recorded at 4.5 minutes into each exercise bout and blood pressure was measured between minutes 6-7. A fingertip capillary blood sample was collected immediately following each stage.

Determination of $\dot{V}O_{2\text{ peak}}$

Participants rested for 15 minutes following the 4 stage submaximal test then completed another standardized 10-minute warmup at 100 W at a self-selected cadence. Participants then performed an incremental ramp protocol at the ramp rate of 30 W·min⁻¹ on a cycle ergometer for the determination of $\dot{V}O_{2\text{ peak}}$. Heart rate and breath-by-breath oxygen uptake ($\dot{V}O_2$) were recorded continuously throughout the test (Cortex Metalyser 3B Biophysik, GmbH, Leipzig, Germany). RPE (Borg 1970) was recorded at 1-minute intervals, pre and post-test lactate samples were obtained immediately before the warmup and following the test.

Supplementation protocol

Participants in the FO group consumed 2600mg of EPA and 1800mg of DHA *n*-3 PUFA supplementation by consuming 8 soft gel capsules per day (based on the manufacturer's analytical content) for 8 weeks (Ultimate Omega lemon soft-gels, Nordic Naturals, Watsonville CA). Participants in the SO (placebo) group consumed 8 soft gel capsules per day of organic soybean oil for 8 weeks containing 438.4 mg total omega 3, 4052.8 mg total omega 6, 2023.2 mg, total omega 9 and 25.6mg of omega 3 from fish oil used as a mask. Participants were instructed to consume the soft - gels 3 times per day 3, 3, and 2 with evenly spaced meals. The relative composition of fatty acids in the FO capsules was quantified by carrying out a batch test. Capsules were analysed by gas chromatography and mass spectrometry in the centre for analytical chemistry at Loughborough University.

Table 5.1 Relative composition of EPA and DHA in each individual FO capsule as Determined by Gas Chromatography compared to the manufacturers label

Fatty Acids	Manufacturer mg/ g	Analytical mg/ g	Recovery
EPA	325	257	79%
DHA	225	171	76%

*Dietary manipulation to lower *n*-6 PUFA levels*

A 7-day food record and list of additional frequently consumed meals and snacks was completed by each participant and emailed to the researcher 1 day prior to visit 2. The diet record was analysed using Nutritics University Edition (2014). Participant diets were screened for high *n*-6 PUFA containing items. Individual foods/ snacks and drinks high in *n*-6 PUFA were substituted with low *n*-6 PUFA options designed to minimize dietary intake of linoleic acid (LA) and arachidonic acid (AA), whilst simultaneously increasing *n*-3 PUFA intake through supplementation. The aim was to achieve a reduction of daily *n*-6 PUFA levels to

the target of <2.5% of energy intake, in accordance with evolutionary levels outlined by Macintosh et al, (2012).

Participants in the SO placebo group were instructed to make a few minor dietary changes to create a placebo diet designed to maintain participant's usual *n-6* PUFA intake for 8 weeks. This created a control condition suitable for double blinding. The substituted 7-day diet records were returned to participants and they were asked to follow a similar diet throughout the supplementation period, this was based on assumption that participants would adhere to the controlled diet.

Participants revisited the researcher halfway through the supplementation period to discuss any issues they were having with adherence. Erythrocyte fatty acids levels were analysed to provide evidence of supplementation. Table 5.2 displays participant total dietary intake of *n-3/ n-6* PUFA in the FO and SO group before the dietary manipulation was applied, compared to the diet they were asked to follow during the supplementation period (Mean \pm SD).

Table 5.2 participant total kcal intake and *n-3/ n-6* PUFA levels in the FO and SO group before and after the dietary intervention was applied (Mean \pm SD).

Group	Pre-dietary Manipulation			Post-dietary Manipulation		
	Total Kcal*	<i>n-3</i> (g)	<i>n-6</i> (g)	Total kcal*	<i>n-3</i> (g)	<i>n-6</i> (g)
FO	2393 \pm 424	0.9 \pm 0.5	9.4 \pm 4.2	2221 \pm 348	0.7 \pm 0.2	4.8 \pm 1.7
SO	2820 \pm 449	1.7 \pm 0.7	11.7 \pm 6.9	2740 \pm 436	1.7 \pm 0.8	11.8 \pm 7.0

* Data is expressed as mean values based on an average dietary intake of 7 days.

Visit 3

Half way through the 8-week supplementation period, participants returned to the laboratory for a venous blood sample and fingertip capillary blood sample (DBS).

Participants were asked if they have been in good health status and were adhering to their dietary changes and were happy to continue the study.

Visit 4

Participants attended the laboratory the day following the end of their eight-week supplementation period for post supplementation testing using the same procedures as detailed in visit 2.

Red blood cell isolation and DBS procedures

Please refer to chapter 4 and appendix 1 for detailed procedures for RBC and DBS preparation and analysis. Fatty acid analysis was carried out using gas chromatography and mass spectrometry in the centre for analytical chemistry at Loughborough University.

Statistical Analysis

Two - way repeated measures mixed ANOVAs were employed to determine any differences in Erythrocyte fatty acid content for the fatty acids of interest (LA, AA, EPA, DHA, total *n*-6 PUFA, total *n*-3 PUFA, total saturated and total monounsaturated FA) at three time points pre supplementation, halfway and eight weeks post supplementation between groups.

Three - way repeated measures mixed ANOVAs were carried out to examine differences in physiological variables: $\dot{V}O_2$, TSI%, deoxy-HHb, HR, DBP, SBP, and MAP (calculated using the formula $MAP = SBP + 2 (DBP) / 3$) during the 4 stage submaximal cycling bouts, between groups, pre to post supplementation. Baseline measures were incorporated in the analysis for all variables except for $\dot{V}O_2$ and deoxy-HHb.

Two-way repeated measures mixed ANOVAs were carried out to examine differences in $\dot{V}O_{2\text{ peak}}$, $power_{\text{max}}$, HR_{max} , $\dot{V}O_2/HR_{\text{max}}$, whole blood lactate and

glucose, pre to post the intervention period between groups. Prior to analysis all data residuals were checked for outliers by inspection of boxplots and normal distribution using the Shapiro Wilk test $p = <0.05$). With regard to NIRS analysis, the participant number reduced in the SO group to $N = 6$ due to NIRS measurement error. Specifically no post supplementation recording was obtained for participant 1 and no pre supplementation recording was obtained for participant 15. Therefore, data for both participants was not included for TSI% and deoxy-HHb analysis.

There were two extreme outliers as determined by boxplots (3 box lengths from the edge of the box) for both TSI % and deoxy-HHb data. The outliers were left in the analysis as the statistical outcome was the same with and without them included.

RESULTS

No differences were observed between groups for age, height, body mass, sum of 7 skinfolds or resting values for HR, BP, lactate, glucose, $\dot{V}O_{2\text{ peak}}$ and max power (see table 5.3).

Table 5.3 Participant characteristics

Participant characteristics	FO ($n = 7$)	SO ($n = 8$)	p -value
Age (y)	48 \pm 6	41 \pm 11	0.144
Height (cm)	176.1 \pm 5.5	181.5 \pm 7.4	0.142
Body mass (kg)	74.6 \pm 10.0	79.1 \pm 6.7	0.316
$\dot{V}O_{2\text{ max}}$ ml/ min/ kg	61.7 \pm 9.0	56.0 \pm 6.9	0.187
Power Max (W)	380 \pm 39.9	379 \pm 43.8	0.982
HR (bpm)	55 \pm 8	57 \pm 6	0.537
Systolic BP (mm Hg)	128.4 \pm 11.0	124.8 \pm 15.2	0.606
Diastolic BP (mm Hg)	82.1 \pm 9.8	77.8 \pm 11.2	0.438
Lactate (mmol/L)	1.7 \pm 0.4	1.8 \pm 0.6	0.578
Glucose (mmol/L)	4.3 \pm 0.2	4.3 \pm 0.4	0.863
Sum of 7 skinfolds (mm)	82.5 \pm 33.9	104.7 \pm 16.7	0.125
Energy Intake (kcal/d)	2393 \pm 424	2820 \pm 449	
Total n -3 PUFA (g)	0.9 \pm 0.5	1.7 \pm 0.7	
Total n -6 PUFA (g)	9.4 \pm 4.2	11.7 \pm 6.9	
Proportion of n -6 to n -3 (g)	10.4	6.9	

Note: Data are expressed as mean (\pm SD)

An independent samples t -test elicited no significant differences in participant characteristics between groups at baseline $p > 0.05$

Changes in erythrocyte fatty acid profile over the course of the supplementation/ dietary intervention

For a comprehensive view of all fatty acid values derived from RBC erythrocyte analysis please see table 5.4 and 5.5. Pre-supplementation erythrocyte % fatty acid content (LA, AA, EPA, DHA, total n-6 PUFA, total n-3 PUFA and the ratio of total n-6 PUFA to total n-3 PUFA) was not different between treatment groups. The following is a summary of the main statistical findings.

Erythrocyte LA% content (n-6 PUFA)

A two way mixed ANOVA elicited a significant main effect of time, $F(2, 26) = 7.85$, $p = 0.002$, partial $\eta^2 = 0.38$ and group, $F(1, 13) = 7.018$, $p = 0.020$, partial $\eta^2 = 0.35$. There was a significant interaction between time and group, $F(2, 26) = 6.92$, $p = 0.004$, partial $\eta^2 = 0.35$, on erythrocyte LA % content.

A one way repeated measures ANOVA on each treatment group found the supplementation and dietary intervention elicited significant changes in LA content over time in the FO group $F(2, 12) = 14.16$, $p = 0.001$, partial $\eta^2 = 0.70$ with no significant change observed in the SO group, $F(2, 14) = 1.32$, $p = 0.29$, partial $\eta^2 = 0.16$. Pairwise comparisons in the FO group showed there was an overall decrease in LA concentration pre, (15.17 ± 1.92) to four weeks (11.74 ± 0.92) and to (11.98 ± 1.29) eight weeks post intervention. Post hoc analysis with a Bonferroni adjustment revealed that LA content was significantly decreased from pre intervention to four weeks 3.43 (95% CI, 0.78, 6.07), $p = 0.016$, and from pre intervention to eight weeks post intervention 3.18 (95% CI, 1.38, 4.99), $p = 0.003$, But not from four weeks to eight weeks post intervention 0.24, (95% CI -2.78, 2.31), $p = 1.000$.

Independent samples *t*-tests show LA was significantly lower in the FO group than the SO group at four weeks, -2.94 (95% CI, -4.86 to 1.02), $t(13) = -3.305$, $p = 0.006$ and at eight weeks, -3.91 (95% CI, -6.03 to -1.79), $t(13) = -3.98$, $p = 0.002$.

Erythrocyte AA% content (n-6 PUFA)

A two way mixed ANOVA elicited a significant main effect of time $F(2, 26) = 8.58$, $p = 0.001$, partial $\eta^2 = 0.38$, and no significant main effect of group $F(1, 13) = 2.02$, $p = 0.18$, partial $\eta^2 = 0.14$. There was a significant interaction between time and group, $F(2, 26) = 15.60$, $p < 0.001$, partial $\eta^2 = 0.55$, on erythrocyte AA % content.

A one way repeated measures ANOVA on each treatment group shows the supplementation and dietary intervention elicited significant changes in AA content over time in the FO group, $F(2, 12) = 16.30$, $p < 0.001$, partial $\eta^2 = 0.73$, with no significant change observed in the SO group, $F(2, 14) = 0.85$, $p = 0.45$, partial $\eta^2 = 0.11$. Pairwise comparisons in the FO group showed there was an overall decrease in AA concentration pre (19.99 ± 2.53) to four weeks (17.29 ± 3.45) and to (15.39 ± 1.19) eight weeks (post intervention). Post hoc analysis with a Bonferroni adjustment revealed that AA content was significantly decreased from pre intervention to four weeks 2.70 (95% CI 1.02, 4.39), $p = 0.006$, and from pre intervention to eight weeks post intervention 4.60 (95% CI, 2.05, 7.16), $p = 0.003$, but not from four weeks to eight weeks post intervention 1.90, (95% CI -1.55, 5.35], $p = 0.361$.

Independent samples *t*-tests show AA was significantly lower in the FO group than the SO at eight weeks, -4.22 (95% CI, -6.50 to -1.94), $t(10.09) = -4.12$, $p = 0.002$, but not at four weeks, -1.90 (95% CI, -5.20 to 1.397), $t(13) = -1.25$, $p = 0.24$.

Erythrocyte EPA% content (n-3 PUFA)

A two way mixed ANOVA elicited a significant main effect of time $F(2, 26) = 39.98$, $p < 0.001$, partial $\eta^2 = 0.76$ and group $F(1, 13) = 32.54$, $p < 0.001$, partial $\eta^2 = 0.72$. There was a significant interaction between time and group, $F(2, 26) = 35.99$, $p < 0.001$, partial $\eta^2 = 0.74$, on erythrocyte EPA % content.

A one way repeated measures ANOVA on each treatment group shows the supplementation and dietary intervention elicited significant changes in EPA content over time in the FO group $F(2, 12) = 40.08$, $p < 0.001$, partial $\eta^2 = 0.87$, with no significant change observed in the SO group, $F(2, 14) = 0.67$, $p = 0.45$, partial $\eta^2 = 0.88$. Pairwise comparisons in the FO group showed there was an overall increase in EPA concentration pre, (1.30 ± 0.20) to four weeks (4.66 ± 1.05) and to (5.85 ± 1.68) eight weeks (post intervention). Post hoc analysis with a Bonferroni adjustment revealed that EPA content was significantly increased from pre intervention to four weeks 3.36 (95% CI 2.07, 4.65), $p < 0.001$, and from pre intervention to eight weeks post intervention 4.55 (95% CI, 2.48, 6.63), $p = 0.001$, but not from four weeks to eight weeks post intervention 1.19, (95% CI -0.55, 2.94), $p = 0.198$.

Independent samples t -tests found EPA was significantly higher in the FO group than the SO group at four weeks 2.86 (95% CI, 1.79 to 3.94), $t(13) = 5.74$, $p < 0.001$, and at eight weeks, 4.23 (95% CI, 2.64 to 5.83), $t(8.56) = 6.04$, $p < 0.001$.

Erythrocyte DHA% content (n-3 PUFA)

A two way mixed ANOVA elicited no significant main effect of time $F(2, 26) = 0.97$, $p = 0.39$, partial $\eta^2 = 0.069$ or group $F(1, 13) = 3.09$, $p = 0.10$, partial $\eta^2 = 0.192$. There was a significant interaction between time and group, $F(2, 26) = 8.99$, $p = 0.001$, partial $\eta^2 = 0.41$, on erythrocyte DHA % content.

A one way repeated measures ANOVA on each treatment group shows the supplementation and dietary intervention elicited significant changes in DHA content over time in the FO group $F(2, 12) = 5.22$, $p = 0.023$, partial $\eta^2 = 0.47$, with no significant change observed in the SO group, $F(2, 14) = 4.41$, $p = 0.072$, partial $\eta^2 = 0.39$. Pairwise comparisons in the FO group showed there was an overall increase in DHA concentration pre, (4.97 ± 2.82) to four weeks (7.12 ± 2.86) and to (9.35 ± 1.12) eight weeks post intervention. Post hoc analysis with a Bonferroni adjustment revealed that DHA content significantly increased from pre intervention to eight weeks post intervention 4.38 (95% CI, 0.51, 8.24), $p = .029$, but not pre intervention to 4 weeks 2.147 (95% CI, -3.58, 7.874), $p = 0.79$, or from 4 weeks to 8 weeks post intervention 2.23, (95% CI, -1.20, 5.66), $p = 0.23$.

Independent samples t -tests show DHA was significantly higher in the FO group than the SO group at week eight, 5.34 (95% CI, 3.09 to 7.59), $t(13) = 5.12$, $p < 0.001$, but not at week four, 0.14 (95% CI, -2.74 to 3.03), $t(13) = 0.11$, $p = 0.92$.

Erythrocyte total n-6 PUFA content

A two way mixed ANOVA elicited a significant main effect of time $F(2, 26) = 16.627$, $p < 0.001$, partial $\eta^2 = 0.561$ and group $F(1, 13) = 16.48$, $p = 0.001$, partial $\eta^2 = 0.56$. There was a significant interaction between time and group, $F(2, 26) = 14.91$, $p < 0.001$, partial $\eta^2 = 0.53$, on erythrocyte total n-6 PUFA % content

A one way repeated measures ANOVA on each treatment group shows the supplementation and dietary intervention elicited significant changes in total n-6 PUFA content over time in the FO group $F(2, 12) = 21.96$, $p < 0.001$, partial $\eta^2 = 0.79$, with no significant change observed in the SO group, $F(2, 14) = 1.74$, $p = 0.21$, partial $\eta^2 = 0.20$. Pairwise comparisons in the FO group showed there was an overall decrease in n-6 PUFA concentration pre, (40.27 ± 1.85) to four weeks (33.02 ± 4.82) and to (30.75 ± 1.29) eight weeks (post intervention). Post hoc analysis with a Bonferroni adjustment revealed that total n-6 PUFA content significantly decreased from pre intervention to four weeks 7.23 (95% CI, -2.78, 11.73), $p = 0.005$, and pre intervention to eight weeks 9.52 (95% CI, 6.15, 12.89), $p < 0.001$, but not from four weeks to eight weeks post intervention 2.26, (95% CI, -4.19, 8.71), $p = 0.88$.

Independent samples t -tests show total n-6 PUFA was significantly lower in the FO group than the SO group at four weeks, -5.74 (95% CI, -10.52 to -.97), $t(13) = -2.60$, $p = 0.022$, and at eight weeks, -9.86 (95% CI, -13.18 to -6.54), $t(13) = -6.42$, $p < 0.001$.

Erythrocyte total n-3 PUFA content (n-3)

A two way mixed ANOVA elicited a significant main effect of time $F(2, 26) = 7.61, p = 0.002$, partial $\eta^2 = 0.37$ and group $F(1, 13) = 18.12, p = 0.001$, partial $\eta^2 = 0.58$. There was a significant interaction between time and group, $F(2, 26) = 19.14, p < 0.001$, partial $\eta^2 = 0.60$, on erythrocyte total n-3 PUFA % content.

A one way repeated measures ANOVA on each treatment group shows the supplementation and dietary intervention elicited significant changes in total n-3 PUFA content over time in the FO group $F(2, 12) = 17.02, p < 0.001$, partial $\eta^2 = 0.74$, with no significant change observed in the SO group, $F(2, 14) = 3.99, p = 0.42$, partial $\eta^2 = 0.36$. Pairwise comparisons in the FO group showed there was an overall increase in n-3 PUFA concentration pre, (10.43 ± 2.53) to four weeks (16.18 ± 2.99) and to (20.13 ± 2.83) eight weeks (post intervention). Post hoc analysis with a Bonferroni adjustment revealed that total n-3 PUFA content significantly increased from pre intervention to eight weeks 9.70 (95% CI, 4.52, 14.88), $p = 0.003$, but not pre intervention to four weeks 5.75 (95% CI, -0.59, 12.08), $p = 0.74$, and not from four weeks to eight weeks 3.96 , (95% CI -0.91, 8.83), $p = 0.11$.

Independent samples *t*-tests show n-3 PUFA was significantly higher in the FO group than the SO group at week four, 3.67 (95% CI, 0.13 to 7.22), $t(13) = 2.24, p = 0.043$ and at week eight, 10.66 (95% CI, 7.44 to 13.87), $t(13) = 7.15, p = < 0.001$.

Erythrocyte Proportion of n-6 PUFA to n-3 PUFA (n6/ n-3)

A two way mixed ANOVA elicited a significant main effect of time $F(2, 26) = 6.515$, $p = 0.005$ partial $\eta^2 = 0.334$ and group $F(1, 13) = 11.37$, $p = 0.005$, partial $\eta^2 = 0.466$. There was a significant interaction between time and group, $F(2, 26) = 17.59$, $p < 0.001$ partial $\eta^2 = 0.575$, on erythrocyte proportion of n-6 to n-3 PUFA content.

A one way repeated measures ANOVA on each treatment group shows the supplementation and dietary intervention elicited significant changes in proportion of n-6 to n-3 PUFA content over time in the FO group $F(2, 12) = 17.39$, $p < 0.001$, partial $\eta^2 = 0.74$, with no significant change observed in the SO group, $F(1.15, 8.05) = 5.64$, $p = 0.4$, partial $\eta^2 = 0.45$. Pairwise comparisons in the FO group showed there was an overall decrease in the proportion of n-6 to n-3 PUFA concentration pre, (4.12 ± 1.26) to four weeks (2.09 ± 0.46) and to (1.56 ± 0.30) eight weeks (post intervention). Post hoc analysis with a Bonferroni adjustment revealed that the proportion of n-6 to n-3 PUFA significantly decreased from pre intervention to 4 weeks 2.03 (95% CI, 0.17, 3.90), $p = 0.034$, and from pre intervention to eight weeks 2.56 (95% CI, 0.84, 4.28), $p = 0.008$, but not from 4 weeks to eight weeks 5.26, (95% CI -0.11, 1.16), $p = 0.10$.

Independent samples t -tests show the proportion of n-6 to n-3 PUFA was significantly lower in the FO group than the SO group at week four, -1.25 (95% CI, -2.21 to -2.87), $t(13) = -2.81$, $p = 0.015$, and at week eight, -3.08 (95% CI, -4.24 to -1.92), $t(7.741) = -6.166$, $p < 0.001$.

No interactions were observed for Total saturated fat or total monounsaturated fat (see appendix 2)

Table 5.4. Changes in erythrocyte fatty acid content (%) at week 0, week 4 and week 8 of the supplementation period

	FO-pre	FO-4 weeks	FO-post	SO-pre	SO-4weeks	SO-post	<i>p</i> (time)	<i>p</i> (group)	<i>p</i> (time*group)
Methyl tetradecanoate	0.41 ± 0.08	0.38 ± 0.06	0.42 ± 0.13	0.35 ± 0.08	0.36 ± 0.06	0.28 ± 0.13	0.600	0.041	0.323
Methyl myristoleate	0.92 ± 0.28	1.12 ± 0.51	1.22 ± 0.41	1.10 ± 0.28	1.31 ± 0.51	1.28 ± 0.41	0.310	0.298	0.906
Methyl pentadecanoate	0.19 ± 0.09	0.16 ± 0.11	0.25 ± 0.02	0.19 ± 0.09	0.22 ± 0.11	0.16 ± 0.02	0.818	0.642	0.029*
Methyl cis- 10 pentadecanoate	0.27 ± 0.25	0.39 ± 0.50	0.14 ± 0.24	0.28 ± 0.25	0.37 ± 0.50	0.70 ± 0.24	0.488	0.117	0.050
Hexadecanoic acid, methyl ester	24.28 ± 1.51	22.24 ± 3.10	23.32 ± 1.05	23.34 ± 1.51	23.03 ± 3.10	24.33 ± 1.05	0.112	0.608	0.225
cis-10-Heptadecenoic acid, methyl	0.41 ± 0.05	0.41 ± 0.14	0.49 ± 0.12	0.45 ± 0.05	0.51 ± 0.14	0.31 ± 0.12	0.445	0.756	0.062
Methyl stearate	13.31 ± 0.66	12.32 ± 2.38	12.60 ± 2.01	12.56 ± 0.66	12.23 ± 2.38	13.43 ± 2.01	0.498	0.999	0.518
9-Octadecenoic acid (Z)-, methyl ester	7.56 ± 0.85	6.90 ± 0.99	7.08 ± 0.51	7.08 ± 0.85	6.90 ± 0.99	7.54 ± 0.51	0.110	0.982	0.121
LA	15.17 ± 1.92	11.74 ± 0.92	11.98 ± 1.30	15.34 ± 1.92	14.69 ± 0.92	15.90 ± 1.30	0.002**	0.020	0.004**
gamma-Linolenic acid, methyl ester	0.05 ± 0.07	0.02 ± 0.03	0.00 ± 0.00	0.03 ± 0.07	0.04 ± 0.03	0.06 ± 0.00	0.715	0.431	0.062
ALA	0.27 ± 0.06	0.21 ± 0.05	0.20 ± 0.10	0.31 ± 0.06	0.28 ± 0.05	0.23 ± 0.10	0.106	0.181	0.663
cis-11,14-Eicosadienoic acid, methyl	0.29 ± 0.10	0.33 ± 0.05	0.33 ± 0.06	0.22 ± 0.06	0.41 ± 0.05	0.32 ± 0.06	0.441	0.965	0.407
8,11,14-Eicosatrienoic acid, methyl e	2.02 ± 0.61	1.39 ± 0.43	1.28 ± 0.23	2.15 ± 0.61	1.94 ± 0.43	2.07 ± 0.23	0.002**	0.132	0.027*
AA	19.99 ± 2.53	17.29 ± 3.45	15.39 ± 1.19	18.91 ± 2.53	19.19 ± 3.45	19.61 ± 1.19	0.001**	0.178	<0.001**
EPA	1.30 ± 0.20	4.66 ± 1.05	5.85 ± 1.68	1.55 ± 0.20	1.79 ± 1.05	1.62 ± 1.68	<0.001**	<0.001**	<0.001**
DPA	3.89 ± 0.42	4.19 ± 0.95	4.74 ± 0.59	3.42 ± 0.42	3.45 ± 0.95	3.62 ± 0.59	0.035*	0.004	0.267
DHA	4.97 ± 2.82	7.12 ± 2.86	9.34 ± 1.12	6.76 ± 2.82	6.98 ± 2.86	4.01 ± 1.12	0.394	0.102	0.001**
15-Tetracosenoic acid, methyl ester,	1.99 ± 1.17	2.55 ± 1.18	3.63 ± 1.93	2.33 ± 1.17	3.83 ± 1.19	1.92 ± 1.93	0.198	0.948	0.517
cis-7,10,13,16-Docosatetraenoate	2.70 ± 0.68	2.21 ± 0.70	1.74 ± 0.40	2.50 ± 0.68	2.47 ± 0.70	2.63 ± 0.40	0.016*	0.399	0.002**
Total saturated	38.19 ± 1.99	35.10 ± 5.44	36.58 ± 2.35	36.45 ± 1.99	35.83 ± 5.44	38.20 ± 2.35	0.159	0.825	0.303
Total Monosaturated	11.15 ± 1.01	11.01 ± 2.41	12.56 ± 1.58	11.23 ± 1.01	12.93 ± 2.41	11.74 ± 1.58	0.216	0.472	0.066
Total <i>n</i> -6	40.27 ± 1.85	33.02 ± 4.82	30.75 ± 1.29	40.31 ± 1.85	38.76 ± 4.82	40.61 ± 1.29	<0.001**	<0.001**	<0.001**
Total <i>n</i> -3	10.43 ± 2.53	16.18 ± 3.00	20.13 ± 2.83	12.04 ± 2.53	12.50 ± 3.00†	9.48 ± 2.83	0.002**	<0.001**	<0.001**
Total <i>n</i> -6/ total <i>n</i> -3	4.12 ± 1.17	2.09 ± 0.43	1.56 ± 0.28	3.52 ± 0.88	3.34 ± 1.09	4.64 ± 1.37	0.005**	0.005**	<0.001**
Total saturated	38.19 ± 1.99	35.09 ± 5.44	36.58 ± 2.35	33.02 ± 9.19	33.18 ± 7.99	35.10 ± 9.61	0.351	0.657	0.257
Total monounsaturated	11.15 ± 1.01	11.01 ± 2.41	12.56 ± 1.58	11.23 ± 1.80	12.93 ± 1.61	11.74 ± 1.03	0.188	0.597	0.333

Note: Data are expressed as means ± SD. **p* < 0.05; ***p* < 0.01 two-way ANOVA interaction

Table 5.5 Changes in erythrocyte fatty acid content % (main fatty acids of focus) at pre, week 4 and week 8 of the supplementation period

		2 way mixed ANOVA main effects					
		Week 0 (pre)	Week 4	Week 8 (post)	<i>p</i> (time)	<i>p</i> (group)	<i>p</i> (time*group)
LA	FO ††	15.17 ± 1.92	11.74 ± 0.92	11.98 ± 1.30			
LA	SO	15.34 ± 1.92	14.69 ± 0.92 ††	15.90 ± 1.30 ††	0.002**	0.02	0.004**
AA	FO ††	19.99 ± 2.53	17.29 ± 3.45	15.39 ± 1.19			
AA	SO	18.91 ± 2.53	19.19 ± 3.45	19.61 ± 1.19 ††	0.001**	0.18	< 0.001**
EPA	FO ††	1.30 ± 0.20	4.66 ± 1.05	5.85 ± 1.68			
EPA	SO	1.55 ± 0.20	1.79 ± 1.05 ††	1.62 ± 1.68 ††	<0.001**	<0.001**	<0.001**
DHA	FO †	4.97 ± 2.82	7.12 ± 2.86	9.34 ± 1.12			
DHA	SO	6.76 ± 2.82	6.98 ± 2.86	4.01 ± 1.12 ††	0.39	0.10	0.001**
Total <i>n</i> -6	FO ††	40.27 ± 1.85	33.02 ± 4.82	30.75 ± 1.29			
Total <i>n</i> -6	SO	40.31 ± 1.85	38.76 ± 4.82 ††	40.61 ± 1.29 ††	<0.001**	0.001**	<0.001**
Total <i>n</i> -3	FO ††	10.43 ± 2.53	16.18 ± 3.00	20.13 ± 2.83			
Total <i>n</i> -3	SO	12.04 ± 2.53	12.50 ± 3.00 ††	9.48 ± 2.83 ††	0.002**	0.001**	<0.001**
<i>n</i> -6/ <i>n</i> -3	FO ††	4.12 ± 1.17	2.09 ± 0.43	1.56 ± 0.28			
<i>n</i> -6/ <i>n</i> -3	SO	3.52 ± 0.88	3.34 ± 1.09 †	4.64 ± 1.37 ††	0.005**	0.005**	<0.001**
Sat	FO	38.19 ± 1.99	35.09 ± 5.44	36.58 ± 2.35			
Sat	SO	33.02 ± 9.19	33.18 ± 7.99	35.10 ± 9.61	0.351	0.657	0.257
Mono	FO	11.15 ± 1.01	11.01 ± 2.41	12.56 ± 1.58			
Mono	SO	11.23 ± 1.80	12.93 ± 1.61	11.74 ± 1.03	0.188	0.597	0.333

Note: Data are expressed as means ± standard deviation

p* < 0.05; *p* < 0.01, two way mixed ANOVA main effects

‡*p* < 0.05; ††*p* < 0.01, one way repeated measures ANOVA main effect of time within groups

†*p* < 0.05; ††*p* < 0.01, Independent *t*-test comparison between groups at wk. 0, wk. 4, wk. 8 . Wk = week of supplementation

The four stage submaximal power test

$\dot{V}O_2$ (L/min) cost of cycling at four submaximal power stages

There was a significant main effect of power level, $F(3, 39) = 40.17$, $p < 0.001$, partial $\eta^2 = 0.76$. There was no significant main effect of pre/ post, $F(1, 13) = 2.06$, $p = 0.18$, partial $\eta^2 = 0.14$ or group, $F(1, 13) = 0.309$, $p = 0.59$, partial $\eta^2 = 0.023$. There was no significant two way interaction between power level and group, $F(3, 39) = 0.82$, $p = 0.49$, partial $\eta^2 = 0.06$, pre/post and group, $F(1, 13) = 0.60$, $p = 0.45$, partial $\eta^2 = 0.04$, or power level *and* pre/post, $F(3, 39) = 0.656$, $p = 0.57$, partial $\eta^2 = 0.048$. Furthermore, there was no significant three way interaction between group, pre/post and power level, $F(3, 39) = 0.829$, $p = 0.49$, partial $\eta^2 = 0.060$ (see table 5.6 and figure 5.2).

Table 5.6 $\dot{V}O_2$ (L/min) values pre and post the intervention in each group at each power level

Stage	Group	Pre	Post	N=
100 W	FO	1.85 \pm 0.24	1.66 \pm 0.08	7
	SO	1.89 \pm 0.44	1.82 \pm 0.44	8
125 W	FO	2.32 \pm 0.26	1.98 \pm 0.11	7
	SO	2.07 \pm 0.32	2.24 \pm 0.54	8
150 W	FO	2.41 \pm 0.58	2.01 \pm 0.25	7
	SO	2.48 \pm 0.42	2.30 \pm 0.60	8
175 W	FO	2.73 \pm 0.47	2.47 \pm 0.15	7
	SO	2.71 \pm 0.36	2.44 \pm 0.65	8

Data is expressed as means \pm SD

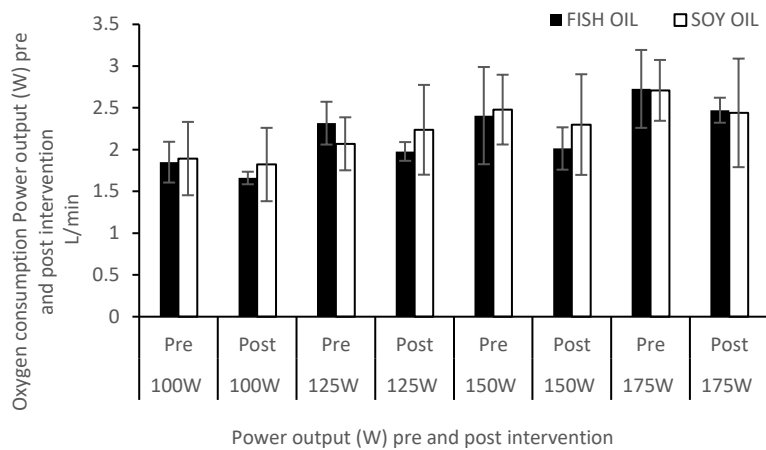


Figure 5.2 $\dot{V}O_2$ (L/ min-1) values pre and post the intervention in FO and SO group.

Data is expressed as means \pm SD.

Heart rate (bpm) at each submaximal power level

There was a significant main effect of power level, $F(1.39, 18.09) = 294.52$, $p < 0.001$, partial $\eta^2 = 0.96$. There was no significant main effect of pre/ post, $F(1, 13) = 0.003$, $p = 0.960$, partial $\eta^2 = <0.001$, or group, $F(1, 13) = 0.427$, $p = 0.53$, partial $\eta^2 = 0.03$. There was no significant two-way interaction between power level and group, $F(1.39, 18.09) = 0.009$, $p = 0.968$, partial $\eta^2 = 0.001$, pre/post and group $F(1, 13) = 0.20$, $p = 0.89$, partial $\eta^2 = 0.002$, or pre/post and power level $F(2.59, 33.642) = 2.576$, $p = 0.078$, partial $\eta^2 = 0.165$. Furthermore, there was no significant three way interaction between group, pre/post and power level, $F(2.59, 33.64) = 2.24$, $p = 0.11$, partial $\eta^2 = 0.15$ (see table 5.7 and figure 5.3).

Table 5.7. HR (bpm) pre and post intervention in the FO and SO group at each power level (including resting measures)

Stage	Group	Pre	Post	<i>N</i>
Baseline	FO	54.9 ± 7.74	54.4 ± 7.53	7
	SO	57.2 ± 6.24	60.4 ± 6.53	8
100 W	FO	99.9 ± 9.2	103.6 ± 6.34	7
	SO	106.4 ± 15.6	104.5 ± 13.2	8
125 W	FO	109.1 ± 14.1	111.4 ± 6.32	7
	SO	113.4 ± 15.0	115.3 ± 20.3	8
150 W	FO	122.1 ± 9.7	119.8 ± 5.70	7
	SO	125.7 ± 17.1	124.0 ± 17.3	8
175 W	FO	134.5 ± 12.9	129.4 ± 6.57	7
	SO	135.5 ± 19.2	135.0 ± 18.3	8

Data is expressed as means ± SD.

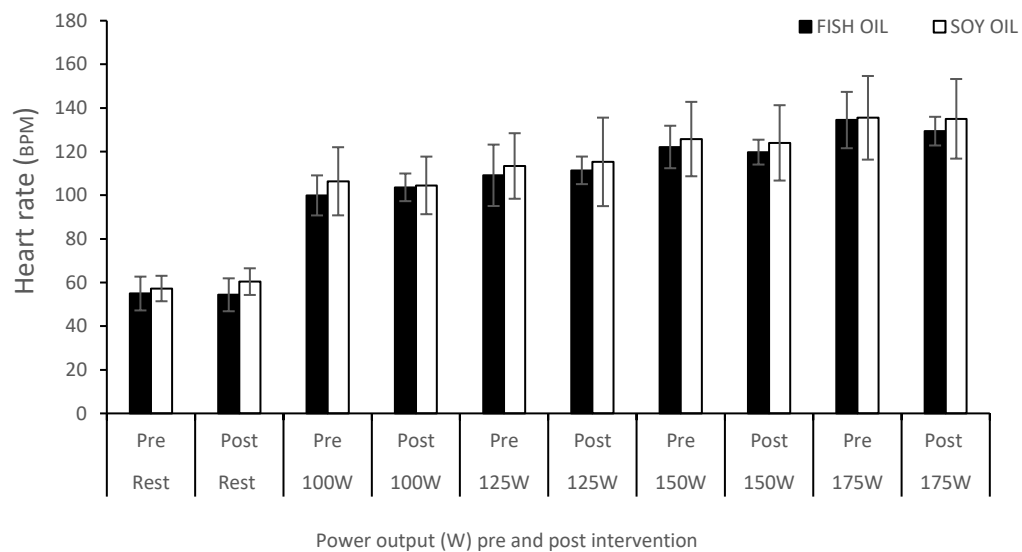


Figure 5.3 HR (bpm) values pre and post the intervention in FO and SO

Data is expressed as means ± SD.

Tissue saturation index (TSI %) for each power level of the four stage submaximal oxygen consumption test

There was a significant main effect of power level, $F(1.46, 16.04) = 13.01$, $p = 0.001$, partial $\eta^2 = 0.54$. There was no significant main effect of pre/ post, $F(1, 11) = 2.541$, $p = 0.139$, partial $\eta^2 = 0.18$ or group, $F(1, 11) = 0.015$, $p = 0.90$, partial $\eta^2 = 0.001$. There was no significant two-way interaction between power level and group, $F(1.46, 16.04) = 0.23$, $p = 0.72$, partial $\eta^2 = 0.020$, pre/post and group, $F(1, 11) = 0.078$, $p = 0.79$, partial $\eta^2 = 0.007$ or power level and pre/ post $F(1.85, 20.38) = 0.15$, $p = 0.85$, partial $\eta^2 = 0.013$. Furthermore, there was no statistically significant three way interaction between group, pre/ post and power level, $F(1.85, 20.38) = 0.87$, $p = 0.43$, partial $\eta^2 = 0.073$ (see table 5.8 and figure 5.4).

Table 5.8. TSI percentage pre and post the supplementation period in the FO and SO group at each power level

Stage	Group	Pre	Post	N
Baseline	FO	68.01 \pm 4.43	68.45 \pm 4.33	7
	SO	67.07 \pm 3.61	70.50 \pm 4.73	6
100 W	FO	65.16 \pm 5.57	67.51 \pm 5.29	7
	SO	65.37 \pm 2.94	67.66 \pm 5.66	6
125W	FO	64.99 \pm 4.49	65.93 \pm 6.20	7
	SO	63.83 \pm 2.10	66.06 \pm 4.57	6
150 W	FO	63.76 \pm 5.62	64.94 \pm 6.59	7
	SO	62.91 \pm 2.01	64.82 \pm 7.14	6
175 W	FO	61.16 \pm 5.75	64.16 \pm 6.08	7
	SO	60.90 \pm 2.55	62.27 \pm 7.96	6

Data is expressed as means \pm SD

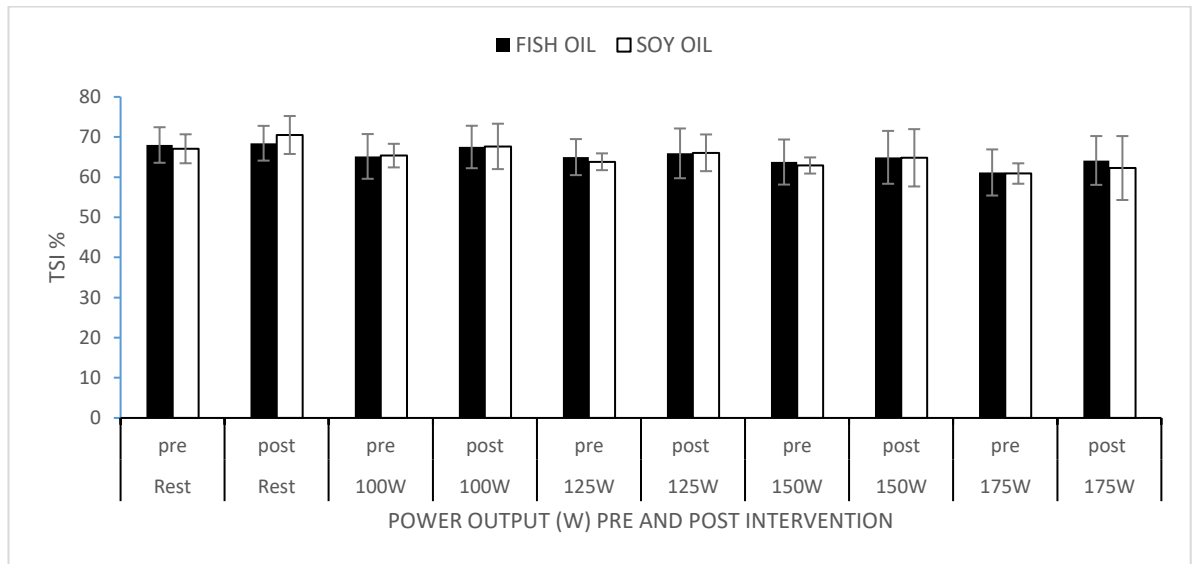


Figure 5.4 TSI% values pre and post the intervention in FO and SO.

Data is expressed as means \pm SD

Deoxygenated haemoglobin deoxy - HHb for each power level of the four-stage submaximal oxygen consumption test. Data is minus baseline levels

There was a significant main effect of power $F(1.18, 12.98) = 16.02, p = 0.001$, partial $\eta^2 = 0.59$. There was no significant main effect of pre/ post, $F(1, 11) = 2.10, p = 0.175$, partial $\eta^2 = 0.16$ or group, $F(1, 11) = 0.35, p = 0.566$, partial $\eta^2 = 0.31$. There was no statistically significant two-way interaction between power level and group, $F(1.18, 12.98) = 0.45, p = 0.55$, partial $\eta^2 = 0.04$, or pre/post and group, $F(1, 11) = 2.16, p = 0.17$, partial $\eta^2 = 0.16$. There was a statistically significant two-way interaction between power level & pre/ post, $F(3, 33) = 3.303, p = 0.032$, partial $\eta^2 = 0.23$. There was no statistically significant three way interaction between group, pre/ post and power level, $F(3, 33) = 0.70, p = 0.56$, partial $\eta^2 = 0.06$ (see table 5.9 and figure 5.5). Post-hoc follow up of the two-way power level and pre/post interaction was carried out using paired samples t -tests (with a Bonferroni correction) There were no significant differences between pre and post HHb values at each power intensity (see table 5.10).

Table 5.9 deoxy-HHb pre and post supplementation in the FO and SO group at each power level

Stage	Group	Pre	Post	N
100 W	FO	4.19 ± 4.61	1.67 ± 4.97	7
	SO	3.49 ± 9.06	4.50 ± 5.96	6
125 W	FO	5.23 ± 4.44	2.18 ± 3.24	7
	SO	5.31 ± 8.52	5.59 ± 6.63	6
150 W	FO	7.60 ± 5.78	3.03 ± 4.02	7
	SO	7.64 ± 9.70	7.17 ± 8.58	6
175 W	FO	10.48 ± 5.24	3.88 ± 4.37	7
	SO	10.40 ± 10.72	9.70 ± 9.05	6

Data is expressed as means ± SD. Data for each power level is corrected to baseline calculated by: minute average during exercise minus minute average at baseline

Table 5.10 Post Hoc paired samples *t*-test to examine differences in pre/ post deoxy - HHb values over four power levels

Pre/ post stage	Mean	Standard deviation	95% CI of the Difference		t	df	Significance (2 tailed)
			Lower	Upper			
Pre/Post 100 W	0.34	4.89	-2.77	3.45	0.24	11	<i>p</i> = 0.81
Pre/Post 125 W	1.29	5.21	-2.01	4.60	0.86	11	<i>p</i> = 0.41
Pre/Post 150 W	2.63	6.73	-1.65	6.91	1.35	11	<i>p</i> = 0.20
Pre/Post 175 W	3.79	6.80	-0.53	8.11	1.93	11	<i>p</i> = 0.08

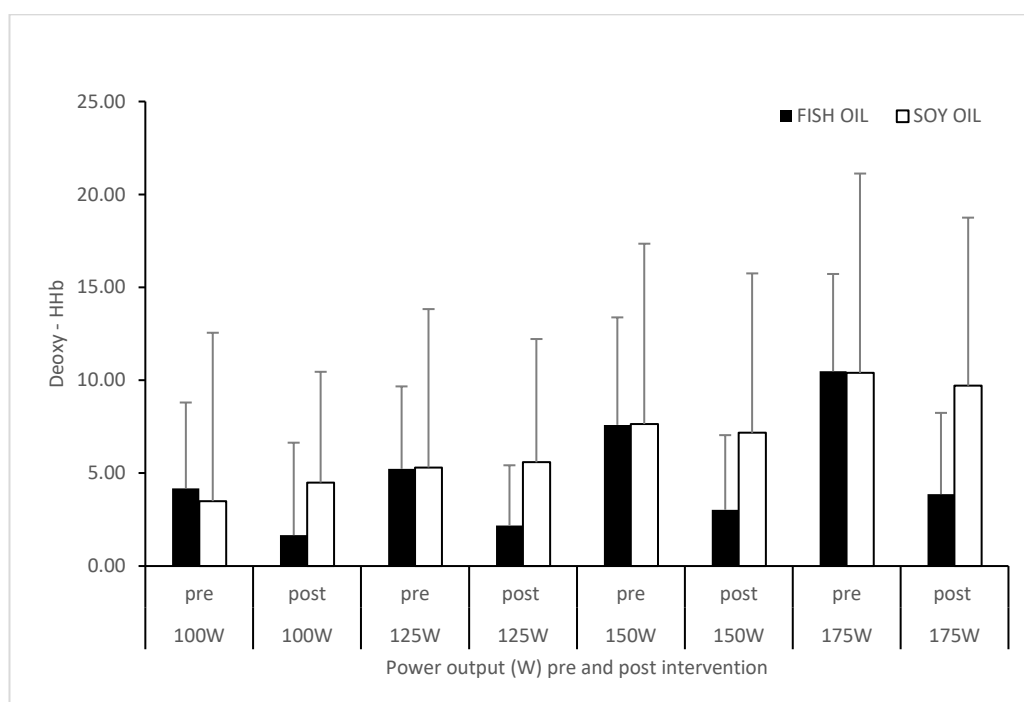


Figure 5.5 deoxy- HHb values pre and post the intervention in FO and SO.

Data is expressed as means ± SD. Data for each power level is corrected to baseline calculated by: minute average during exercise minus minute average at baseline.

Systolic BP for each power level of the four-stage submaximal oxygen consumption test (Data should be treated with caution due to tango malfunction halfway through testing for all participants error of measurement was in both directions)

There was a significant main effect of power level, $F(4, 52) = 31.83, p < 0.001$, partial $\eta^2 = 0.710$. There was no significant main effect of pre/ post, $F(1, 13) = 3.56, p = 0.08$, partial $\eta^2 = 0.22$, or group, $F(1, 13) = 4.628, p = 0.051$, partial $\eta^2 = 0.263$. There was no significant two-way interaction between power level & group, $F(4, 52) = 2.16, p = 0.09$, partial $\eta^2 = 0.14$. Pre/post and group $F(1, 13) = 3.65, p = 0.08$, partial $\eta^2 = <0.21$, or pre/post and power level $F(4, 52) = 2.13, p = 0.09$, partial $\eta^2 = 0.14$ (see table 5.11).

Table 5.11 Systolic BP (mm/Hg) pre and post the supplementation period in the FO and SO group at each power level

Stage	Group	Pre	Post	N
Baseline	FO	128.4 ± 11.0	128.6 ± 22.3	7
	SO	124.8 ± 15.2	131.1 ± 14.8	8
100 W	FO	174.0 ± 23.8	161.7 ± 21.4	7
	SO	162.7 ± 20.9	141.6 ± 34.1	8
125 W	FO	176.6 ± 18.6	188.5 ± 27.2	7
	SO	169.1 ± 23.5	133.7 ± 30.1	8
150 W	FO	186.3 ± 19.0	204.9 ± 31.9	7
	SO	186.4 ± 30.7	152.5 ± 42	8
175 W	FO	200.6 ± 20.0	183.0 ± 48.5	7
	SO	191.3 ± 28.5	158.6 ± 32	8

Note: Data is expressed as means ± SD

Diastolic BP for each power level of the four stage submaximal oxygen consumption test (Data should be treated with caution due to tango malfunction halfway through testing with all participants).

There was no significant main effect of power, $F(4, 52) = 1.63$, $p = 0.180$, partial $\eta^2 = 0.11$, pre/ post, $F(1, 13) = 0.008$, $p = 0.23$, partial $\eta^2 = 0.001$ or group $F(1, 13) = 1.63$, $p = 0.08$, partial $\eta^2 = 0.21$. There was no two-way interaction between power level & group, $F(4, 52) = 2.45$, $p = 0.057$, partial $\eta^2 = 0.16$, pre/post and group $F(1, 13) = <0.001$, $p = 1.00$ partial $\eta^2 <0.001$, or pre/post and power level $F(4, 52) = 0.77$, $p = 0.55$, partial $\eta^2 = 0.06$. Furthermore, there was no three way interaction between power level, pre/post and group, $F(4, 52) = 1.72$, $p = 0.16$, partial $\eta^2 = 0.12$ (see Table 5.12).

Table 5.12. Diastolic BP (mm/Hg) pre and post the supplementation period in the FO and SO group at each power level

Stage	Group	Pre	Post	N
Baseline	FO	82.1 ± 9.84	80.1 ± 11.33	7
	SO	77.8 ± 11.23	80.5 ± 12.2	8
100 W	FO	81 ± 11.1	94.3 ± 18.3	7
	SO	70.7 ± 12.8	68.6 ± 8.1	8
125 W	FO	77.8 ± 4.5	77.0 ± 6.83	7
	SO	69.4 ± 14.2	68.5 ± 23.4	8
150 W	FO	81.1 ± 15.1	75.0 ± 8.90	7
	SO	70.4 ± 14.2	74.3 ± 16.3	8
175 W	FO	76.0 ± 15.8	72.8 ± 8.8	7
	SO	76.0 ± 15.3	73.6 ± 11.0	8

Note: Data is expressed as means ± SD.

Mean arterial pressure (mmHg)

There was a significant main effect of power level, $F(4, 52) = 7.54$, $p < 0.001$ partial $\eta^2 = 0.37$, and group, $F(1, 13) = 5.19$, $p = 0.04$ partial $\eta^2 = 0.285$. The dietary intervention group had a significantly higher MAP on average than the placebo group $p = 0.04$. There was no significant main effect of pre/ post, $F(1, 13) = 1.133$, $p = 0.31$ partial $\eta^2 = 0.08$. There was no significant 2 way interaction between power level and group $F(4, 52) = 2.40$, $p = 0.62$ partial $\eta^2 = 0.16$, pre/post and group $F(1, 13) = 1.26$, $p = 0.28$ partial $\eta^2 = 0.09$ or power level and pre/ post, $F(4, 52) = 1.38$, $p = 0.25$ partial $\eta^2 = 0.10$. Furthermore, there was no significant three way interaction between group, pre/post & power level, $F(4, 52) = 1.36$, $p = 2.59$ partial $\eta^2 = 0.095$ (see table 5.13).

Table 5.13 Mean arterial pressure (MAP mmHg) pre and post the supplementation period in the FO and SO group at each power level

Stage	Group	Pre	Post	<i>N</i>
Rest	FO	97.6 ± 9.5	96.3 ± 14.4	7
	SO	93.4 ± 12.4	97.3 ± 8.6	8
100 W	FO	112 ± 11.8	116.8 ± 16.1	7
	SO	101.4 ± 12.9	92.9 ± 14.21	8
125 W	FO	114.3 ± 13.5	114.2 ± 9.0	7
	SO	102.6 ± 13.9	90.2 ± 24.1	8
150 W	FO	116.2 ± 13.8	124.5 ± 23.1	7
	SO	109 ± 16.1	100.3 ± 19.4	8
175 W	FO	117.5 ± 15.3	114.2 ± 21.7	7
	SO	114.4 ± 18.3	101.9 ± 13.9	8

Note: Data is expressed as means ± SD

There were no effects observed in the four-stage power test between group's pre to post test for RPE, whole blood glucose and lactate levels. A summary of these statistics is in appendix 3.

Maximal oxygen consumption test

$$\dot{V}O_{2\max}$$

A two way mixed ANOVA elicited no significant main effect of pre/ post, $F(1, 13) = 0.034$, $p = 0.86$, partial $\eta^2 = 0.003$, or group $F(1, 13) = 1.672$, $p = 0.22$, partial $\eta^2 = 0.11$. There was no two-way interaction between pre/post and group, $F(1, 13) = 0.35$, $p = 0.56$, partial $\eta^2 = 0.026$ (see table 5.14).

$$HR_{\max}$$

A two way mixed ANOVA showed there was no significant main effect of pre/post, $F(1, 13) = 0.15$, $p = 0.71$ partial $\eta^2 = 0.01$, or group $F(1, 13) = 0.003$, $p = 0.95$ partial $\eta^2 = <0.001$. There was no significant interaction between pre/post and group, $F(1, 13) = 0.05$, $p = 0.83$ partial $\eta^2 = 0.004$ (table 5.14).

$$\dot{V}O_{2\max}/HR_{\max}$$

(1 participant was missing a data point in the SO group so was removed from the analysis.) A two way mixed ANOVA showed there was no statistically significant main effect of pre/post, $F(1, 12) = 0.31$, $p = 0.59$ partial $\eta^2 = 0.03$ or group $F(1, 12) = 0.11$, $p = 0.31$ partial $\eta^2 = 0.09$. There was no significant interaction between pre/post*group $F(1, 12) = 0.12$, $p = 0.91$ partial $\eta^2 = 0.001$ (table 5.14).

$$Power_{\max}$$

A two way mixed ANOVA showed there was no significant main effect of (pre/post), $F(1, 12) = 0.43$, $p = 0.52$ partial $\eta^2 = 0.04$ or group $F(1, 12) = 0.05$, $p = 0.82$, partial $\eta^2 = 0.004$. There was no significant interaction between pre/post and group $F(1, 12) = 1.36$, $p = 0.27$, partial $\eta^2 = 0.10$ (table 5.14).

Table 5.14. Maximal test data pre and post intervention in the FO and SO group

Dependent variable		Pre	Post	<i>N</i>
$\dot{V}O_{2\max}$ (ml/ kg/ min)	FO	61.7 ± 9.0	61.0 ± 7.7	7
	SO	56.0 ± 56.4	56.4 ± 8.1	8
HR _{max} (bpm)	FO	180 ± 9.2	179 ± 6.8	7
	SO	181 ± 20.2	179 ± 11.7	8
$\dot{V}O_{2\max}/HR_{\max}$	FO	0.34 ± 0.05	0.34 ± 0.41	7
	SO	0.31 ± 0.035	0.31 ± 0.05	7
Power _{max} (W)	FO	380 ± 39.9	383 ± 41.8	7
	SO	383 ± 45.3	370 ± 44.4	7

Note: Data is expressed as means ± standard deviation

DISCUSSION

The results of this study demonstrate that an 8-week dietary intervention designed to lower *n*-6 and raise *n*-3 PUFA intake (H3, L6), significantly increased erythrocyte percentage content of total *n*-3 PUFA, EPA and DHA in trained cyclists. The intervention significantly reduced erythrocyte percentage content of total *n*-6 PUFA, LA and AA. In addition, the proportion of *n*-6 to *n*-3 PUFA was significantly reduced. Contrary to the hypothesis, the dietary intervention did not lower the $\dot{V}O_2$ cost of submaximal cycling over four power stages. No changes were observed between groups in measurement of local muscle oxygenation (TSI %), and oxygen extraction (deoxy-HHb). Furthermore, no changes in BP or HR were observed. No changes were observed between groups for $\dot{V}O_{2\text{ peak}}$, $\text{power}_{\text{max}}$, HR_{max} or $\dot{V}O_{2\text{ peak}}/\text{HR}_{\text{max}}$ in the incremental cycling test to exhaustion. Therefore, although this study demonstrates a successful dietary intervention to increase *n*-3 PUFA incorporation into erythrocytes, *n*-3 PUFA supplementation did not elicit any changes in cardiovascular variables in submaximal cycling.

Integrated Dietary intervention and Erythrocyte percentage content of FA

N-6 LA and total *n*-6 PUFA significantly decreased in the H3 - L6 group over the 8-week intervention period with no change observed in the placebo group. The decrease in the H3/ L6 group was significant from baseline to 4 weeks (and from baseline to 8 weeks but not from 4 weeks to 8 weeks. This is indicative of the reduction being more marked in the first 4 weeks of the intervention period and plateauing thereafter. Therefore, a 4-week intervention period might have been sufficient to lower LA & *n*-6 PUFA tissue levels. However, it is important to consider that the time course of *n*-6 PUFA reduction in specific tissues might vary.

Hodson et al., (2008), reported that adipose tissue *n*-6 LA has a slow turnover rate with a half-life of approximately 1 – 2 years. This suggests a dietary intervention designed to lower LA may need to be maintained for an extended period in order to achieve maximal alterations in some tissues in the human body.

AA significantly reduced over the intervention period and took eight weeks to do so. These findings are in agreement with Macintosh et al. (2013) who reported that 12 weeks of a H3-L6 diet reduced erythrocyte fatty acid levels of *n*-6, LA and AA. It was concluded the reduction in AA might be a result of the Low *n*-6 PUFA diet or raised EPA and DHA levels competing for incorporation into the relevant target tissues by preventing the conversion of ALA reducing the elongation and saturation of *n*-6 PUFA.

In the current study, *n*-3 EPA significantly increased in the H3-L6 group over the 8-week intervention period, with no change observed in the placebo group. The increase was significant from baseline to 4 weeks and from baseline to 8 weeks but not from 4 weeks to 8 weeks. This indicates the increase in EPA was more marked in the first 4 weeks of the intervention period, and plateaued thereafter. Therefore, 4 weeks of *n*-3 PUFA supplementation may be sufficient to raise EPA tissue levels. DHA and total *n*-3 PUFA took 8 weeks to reach significantly increased levels in the H3-L6 group. In the current study, 8 weeks of supplementation (2.6g EPA and 1.8g DHA) increased EPA erythrocyte levels by 350% and DHA by 88%. The large increase in EPA compared to DHA is in agreement with previous studies. Raastad et al. (1997), reported that 10 weeks of *n*-3 PUFA supplementation (1.6g EPA and 1g of DHA per day), increased erythrocyte EPA levels by 175% and DHA by 40%. 4 weeks of *n*-3 PUFA supplementation (0.1g/ kg of supplement per day, equating to a mean intake of 4.9g EPA and 1.4g DHA per day), increased EPA levels by 600% and DHA by 37% (Gravina et al., 2017). Clearly, the dosage of omega 3 and the time course of

supplementation dictates the cellular incorporation of EPA and DHA. This suggests that studies focusing on increasing cellular saturation of DHA may need at least 8 weeks of supplementation when using similar supplement concentrations of EPA and DHA to the current study.

In conclusion the High *n*-3: Low *n*-6 PUFA intervention significantly increased EPA, DHA, total *n*-3 PUFA erythrocyte levels and significantly reduced total *n*-6, LA and AA, over the eight-week dietary intervention. No changes observed in the placebo group. This suggests that participant compliance to H3-L6 was successful and in agreement of our study aims. Previous studies have demonstrated that supplementation with *n*-3 PUFA alone (with no reduction of *n*-6 PUFA dietary intake) significantly reduced *n*-6 PUFA erythrocyte incorporation and markedly increased EPA and DHA incorporation (Gravina et al., 2017). In addition it should be noted that Macintosh et al. (2013), showed that LA can be reduced and EPA and DHA increased (+51% and +19% respectively) without *n*-3 PUFA supplementation, simply by lowering dietary *n*-6 PUFA dietary intake over 12 weeks. This suggests that that high *n*-6 PUFA diets might interfere with synthesis and incorporation of EPA and DHA in human tissue. For future research considerations, an additional 3rd group of participants supplementing with *n*-3 PUFA alone and a 4th group following a low *n*-6 PUFA diet (without *n*-3 PUFA supplementation) would provide further evidence regarding the effect of H3-L6.

Although differences in erythrocyte fatty acid content were observed over the intervention period, it is not known whether levels increased in the target tissues including the heart, blood vessels and skeletal muscle. Further exercise studies that examine muscle biopsies for fibre specific fatty acid content and mitochondrial phospholipid fatty acid composition are warranted. McGlory et al. (2014) found that 4 weeks of supplementation with a high daily dose of *n*-3 PUFA (5g/ day similar to the dosage used in the present study) increased both blood and skeletal muscle *n*-

3 PUFA composition. They also noted that changes in *n*-3 PUFA levels in the blood were detectable after 1 week of supplementation however, changes were not detected in skeletal muscle tissue until 2 weeks post supplementation. Therefore, a minimum, 2 weeks may be required to start to see differences in skeletal muscle FA composition. It is clear that *n*-3 PUFA composition of the tissues is related to dose response and the duration of the supplementation period. There is a need for future research to examine careful systematic *n*-3 PUFA dose response relationship studies that investigate a number of different tissues and their uptake of *n*-3 PUFA.

The effect of the high n-3/ low n-6 PUFA intervention on $\dot{V}O_2$ max and submaximal physiological variables $\dot{V}O_2$, TSI%, BP, HR, RPE, lactate and Glucose

Contrary to the hypothesis, the H3:L6 dietary intervention had no effect on $\dot{V}O_2$, HR, TSI%, deoxy-HHb, BP or MAP during steady state cycling at four submaximal power outputs. Previous studies have reported similar findings to the current study outcome. Hingley et al. (2017) reported there was no effect of *n*-3 PUFA on oxygen consumption at rest or during low intensity submaximal exercise. However, reduced oxygen consumption was observed in the first 2 minutes of a 5-minute cycling time trial in the fish oil group. In contrast, 8g/d of DHA rich fish oil lowered the oxygen cost of cycling to failure (Peoples et al., 2008). This suggests that the *n*-3 PUFA mechanism may be revealed more so under conditions of increased physiological strain when oxygen delivery to the muscles is compromised, or due to cardiovascular changes. Another possibility is the *n*-3 PUFA mechanism might work at a local level improving skeletal muscle $M\dot{V}O_2$ (as has been observed in the myocardium of rats fed with an *n*-3 PUFA diet (Pepe, 2002). In humans, DHA is preferentially incorporated into skeletal muscle cell membranes (Peoples et al.,

2008), (Henry, Peoples, & McLennan, 2015) and may be associated with increased in ADP sensitivity (Herbst et al, 2014). This may be beneficial in athletes that already have a high mitochondrial content and may be reaching their ceiling regarding further training induced adaptations.

Previous researchers examining *n*-3 PUFA and its effect on oxygen cost of exercise have based their findings on $\dot{V}O_2$ or $\dot{V}O_{2\text{ max}}$ measurements (Raastad et al., 1997; Zebrowska et al., 2004; Peoples et al., 2008; Da Boit et al., 2015). Whole body O_2 consumption measured via expired gases closely reflects O_2 consumption in the active muscles during steady state light to moderate exercise (Grassi 1996). Therefore, non-invasive breath-by-breath measurements or Douglas bags are commonly used as a proxy measurement of muscle oxygen uptake ($M\dot{V}O_2$). Direct measurements of $M\dot{V}O_2$ are technically difficult and invasive in nature however, inferences on the relationship between $\dot{V}O_2$ and $M\dot{V}O_2$ can be made using non-invasive muscle oxygenation measurement techniques. This is the first *n*-3 PUFA and exercise related study to use near infrared spectroscopy (NIRS) in addition to $\dot{V}O_2$ measurement. NIRS provides additional information regarding local oxygen delivery and extraction within the tissue region of interest (De Lorey et al, 2003). In the current study tissue saturation index (TSI%) and deoxy- HHb (a measure of muscle oxygen extraction) were examined using NIRS. There was no significant difference in TSI % or deoxy-HHb observed between groups, pre/ post supplementation, during steady state exercise at any of the four power outputs. This further confirms and compliments our findings regarding no significant change in $\dot{V}O_2$ measurement using Douglas bags.

In the current study a diet rich in *n*-3 PUFA was not associated with any significant differences in $\dot{V}O_{2\text{ peak}}$, $\text{power}_{\text{max}}$, HR_{max} or $\dot{V}O_2/\text{HR}_{\text{max}}$ during the ramp based incremental test to exhaustion. This finding is in agreement with previous exercise studies (Oostenbrug et al., 1997; Raastad et al, 1997; Kawabata, 2014; Hingley 2017). In contrast, Zebrowska et al. (2014), found that 3 weeks of omega 3 fatty acid supplementation increased individual $\dot{V}O_{2\text{ max}}$ and $\dot{V}O_2$ in relation to maximal HR ($\dot{V}O_2/\text{HR}_{\text{max}}$). The improvements were attributed to an *n*-3 PUFA supplement induced increase in serum NO levels. However it should be noted that Zebrowska et al. (2014), used a randomised cross over study design, with a 2-week washout period. Cao et al. (2006) suggested that studies with washout periods should consider 8 – 16 weeks due to EPA and DHA concentrations remaining marginally higher than baseline, 8 weeks after the cessation of supplementation with *n*-3 PUFA.

In the present study there was no difference between the high *n*-3/ low *n*-6 PUFA group and the placebo group in resting HR or HR during steady state exercise at 100, 125, 150 or 175W or HR_{max} in the $\dot{V}O_{2\text{ max}}$ test. Fish oil supplementation has previously resulted in a reduction in resting heart rate during submaximal exercise (Buckley et al., 2009), and during maximal aerobic exercise (Oostenburg et al., 1997; Raastad et al., 1997; Zebrowska et al., 2014). This may be due to *n*-3 PUFA affecting ion channel function in auto rhythmic cells of the heart potentially elongating the diastole phase of the cardiac cycle (McLennan, 2001; Mozaffarian, 2005). A Reduction in exercising blood pressure has also been observed in a couple of previous studies. In both healthy young and older subjects, diastolic BP and MAP were reduced in the *n*-3 PUFA supplemented group, at the onset of isometric handgrip exercise (Clark, et al, 2016). The reduction of BP and MAP may be due to *n*-3 PUFA consumption influencing nitric oxide production and the

blunting of vasoconstriction in response to norepinephrine (Chin et al, 1993). Several studies have reported increased arterial flow mediated dilation (FMD) following *n*-3 PUFA supplementation (Walser, 2006; Zebrowska, 2014). FMD was not measured in the current study.

It would be of interest to establish if the observations already discussed would be different when participants are exercising in a stressed state such as a pre fatigued state or oxygen deficient state where a supplement that may provide physiological compensation can be fully scrutinised. More recently a study by Hingley et al. (2017), demonstrated that increasing the omega -3 PUFA Index of erythrocytes lowered oxygen consumption most notably during the first 2 minutes of a 5 minute intense cycling time trial (although cycling performance did not improve). Hingley supplemented cyclists with DHA rich fish oil (560mg DHA/140mg EPA) for 8 weeks. DHA is a primary fatty acid in mitochondrial phospholipids in skeletal muscle and it has been suggested that *n*-3 PUFA supplementation may affect mitochondrial bioenergetics (Herbst, 2014); this raises the question if *n*-3 PUFA supplementation improves the efficiency of mitochondrial respiration.

Limitations

During this study the Tango BP monitor malfunctioned on a number of occasions during testing. It was replaced with a manual sphygmomanometer, which may have affected the BP recordings for the participants, in particular the post supplementation values. As a result of this, the BP findings in the current study should be treated with caution.

The 8-week dietary intervention was reliant on participant compliance. Participants were monitored, four weeks into the supplementation period, where they were asked to produce a 3-day diet record, to ensure they were adhering to the dietary intervention. This was a burden on participants and not all could complete it to a relevant standard. Macintosh et al, (2013) mitigated this issue by providing dietitian counselling 6 x 45 minutes once every two weeks and participants were provided with a 2-week supply of study foods following each visit. In addition, they had access to web based education materials and diet adherence was monitored using 24 h telephone on six unannounced occasions. Further measures such as those employed by Macintosh may have ensured dietary compliance in the current study.

Conclusion

The novel 8-week dietary intervention designed to raise *n*-3 PUFA intake and reduce *n*-6 PUFA successfully achieved this target alteration. Despite changes in fatty acid profile, there were no changes observed in $\dot{V}O_2$, TSI % or deoxy HHb measurements in the fish oil supplemented group compared to the placebo group. Furthermore, no changes was observed in other physiological variables HR, BP, RPE, MAP, or $\dot{V}O_{2\text{ peak}}$ performance. The findings of this study suggest that *n*-3 PUFA is unlikely to be a supplement that reduces the oxygen cost or increases oxygen extraction during steady state submaximal cycling in trained athletes, nor does it improve maximal oxygen consumption. It would be relevant for future research to consider the *n*-3 PUFA action on high intensity aerobic exercise (Hingley, 2017) or exercise in an oxygen deficient state, where a supplement that may provide physiological compensation could be of benefit.

CHAPTER 6 GENERAL DISCUSSION

This thesis aimed to investigate the potential mechanisms by which increased dietary *n*-3 PUFA consumption might enhance exercise performance and cardiovascular function. *N*-3 PUFA has gained a great deal of interest from sport scientists in the past decade, and is still very much under investigation. There has been significant interest in the anti-inflammatory action of *n*-3 PUFA and prolonged high intensity exercise, which can result in immune dysfunction and transient inflammation. This is part of the normal response to exercise and is characterised partly by the production of anti and pro-inflammatory cytokines (Nieman, 2001, 2005, 2009). Previous studies have attempted to quantify the acute cytokine response to different types of exercise. The majority have focused on exercise modalities known to induce muscle damage or extreme inflammatory responses including, exercise in hot environments (Cosio-Lima et al., 2011; Luk et al., 2016), eccentric exercise (Toft et al., 2002), unaccustomed exercise (Sorichter et al., 2006), marathon running and 250-km road cycling (Camus et al., 1997; Gannon et al., 1997, cited in Brenner et al., 1999). Acute cytokine responses are known to be variable in relation to the duration and intensity of the exercise bout, and the mode of exercise performed (Pedersen and Hoffman-Goetz, 2000). Few studies have investigated the cytokine response to different durations or intensities of cycling. Chapter 3 in this thesis was the first study to examine the plasma cytokine response to two cycling protocols that were time trial and interval based. Previous studies have compared the cytokine response to different modes of exercise. Brenner et al. (1999) reported that 2 hours of submaximal cycling induced acute and significant increases in IL-6 and TNF α , whereas no changes were observed for circuit training (Brenner et al., 1999). IL-1 β , IL-6 and TNF α peaked immediately following 3 hours of cycling and inclined walking (Moldoveanu et al., 2000). It was hypothesised that 1 hour of pre-loaded cycling may result in a more marked inflammatory cytokine response compared to interval cycling. The findings that both cycling protocols

elicited an increase in IL-6 concentration, and the increase being more marked post the 1- hour preloaded time trial compared to the interval trial, confirms the typical IL-6 response to exercise that has been well documented in previous studies (Brenner, 1999; Cox, 2007., Suzuki, 2000). However, apart from the observed IL-6 response, both cycling protocols did not evoke the acute pro-inflammatory response described by Peake et al. (2005), whereby IL-6 is the first cytokine to increase in the circulation during exercise, followed by the classic proinflammatory cytokines IL-1 β and TNF α . This thesis demonstrated that IL-4 (anti – inflammatory) and MCP-1 (a chemokine involved in mediating monocyte chemotaxis) concentrations increased and VEGF decreased, post both trials on average. No changes were observed in IL-1 α , IL-1 β , IL-2, IL-8, IL-10, IFN- γ , or TNF α . These findings are in agreement with Ostapiuk-Karolczuk et al. (2012), who observed elevations in IL-6, IL-4 and MCP-1 post 90 minutes of running (65% $\dot{V}O_{2\max}$), elevations in IL-1 β and TNF α were not detected until 6 hours post exercise. The acute IL-4 increase could be explained by its release from immune cells located in the circulation, whereas pro inflammatory cytokines released locally from the muscle may take longer to increase in plasma. Therefore, a delayed increase in inflammatory cytokines could not be ruled out, although previous research has also reported no change or only small changes in inflammatory cytokines post exercise (Hirose et al., 2004).

Limitations

Only examining the acute post exercise cytokine response in chapter 3 was a limitation in this thesis. Previous research has typically obtained blood samples periodically at 6, 12, 24 and 72 hours post exercise (Brenner et al., 1999). The time course of appearance of inflammatory and anti-inflammatory cytokines means that some peak in the hours following exercise such as IL-8 and TNF α (Pedersen, 2000; Ostapiuk-Karolczuk et al., 2012). It would be relevant therefore, in future research

to consider sampling over the hours and days post exercise. Furthermore, some cytokine values were at or below the minimum detection limit. In these cases the standard minimal value for each cytokine was used for analysis. This was particularly the case for IL-2, IL-10, IFNG, IL1 α and IL1- β . In addition, it has been reported that concentrations of cytokines in peripheral blood plasma may not be indicative of up or down regulation in the tissues (Sprenger et al., 1992). Therefore the combined inclusion of muscle biopsies, urine samples (Sugama et al., 2013; Hirose, 2004) and the measurement of arteriovenous differences across the exercising limb (Steesberg, 2000) might assist us in understanding the cytokine response to exercise more clearly in future research.

In addition to the anti-inflammatory action of *n*-3 PUFA, there has been considerable interest in its effects on the cardiovascular system. *N*-3 PUFA supplementation has been reported to reduce oxygen consumption and improve oxygen economy during submaximal exercise (Kawabata, 2014; Peoples & McLennan, 2010, 2016). These findings may be as a result of key *n*-3 PUFA cardiovascular mechanisms reported to augment stroke volume, cardiac output and reduce systemic vascular resistance (Walser et al, 2008). In addition, *n*-3 PUFA has been reported to reduce red blood cell deformability (Oostenbrug et al., 1997), lower blood pressure and heart rate during submaximal exercise (Buckley et al., 2007). Furthermore, *n*-3 PUFA supplementation elevated serum NO concentrations and enhanced vascular endothelial function, improving blood flow (Zebrowska, 2014; Walser et al., 2006; Wu & Meininger, 2002, 2012). However, there have been few reports of a direct benefit of *n*-3 PUFA supplementation on exercise performance. Only two studies have reported that *n*-3 PUFA supplementation enhanced $\dot{V}O_{2\text{ max}}$ or $\dot{V}O_{2\text{ peak}}$ performance (Leaf and Rauch, 1988, Zebrowska et al., 2014). Therefore, the second main aim of this thesis was to determine if *n*-3 PUFA supplementation reduced oxygen consumption during submaximal cycling or influenced $\dot{V}O_{2\text{ peak}}$

performance (Chapter 5). Prior to chapter 5, it was important to validate methods of measurement for the determination of an individual's fatty acid status in chapter 4. The use of such biomarkers are important in this field. They provide some objective measure of the suitability of a dietary intervention to reach target increases in the tissues and furthermore, it determines if participants have adhered to the intervention. Traditional methods to quantify an individual's fatty acid status involve the isolation of RBCs from venous whole blood samples as a surrogate marker of *n*-3 PUFA tissue incorporation. This is considered by some as a gold standard biomarker for the measurement of *n*-3 PUFA status (Metcalf et al, 2007). However, this method is confounded by limitations such as the requirement for a trained phlebotomist and the difficulty in obtaining multiple samples without the procedure becoming invasive. The use of dried blood spots (DBS) as a convenient, rapid, and inexpensive method of sampling was first employed by Marangoni et al., (2004). Therefore, the aim of the second experimental chapter (chapter 4) was to examine the relationship between whole blood fingertip DBS blood samples and isolated erythrocytes for determining an individual's fatty acid status. Previous studies have compared these methods at one time point for example at rest, without dietary manipulation or supplementation (Bailey-Hall et al, 2008; Bell et al, 2011). This is the first study that has examined the relationship between measurement methods over an 8-week *n*-3 PUFA supplementation period using data from 3 time points: baseline, at 4 weeks and at 8 weeks. Results demonstrated there was poor correlation and lack of agreement between methods for the measurement of the majority of fatty acids apart from EPA ($R^2 = 0.64$, $p = 0.005$), intra-class correlation demonstrated agreement between methods for EPA (ICC = 0.49). In addition, Bland and Altman levels of agreement were good for EPA (mean difference = 1.12% with 95% limits of agreement -1.43% – 3.67% without any evidence of proportional bias $p = 0.05$). Higher mean percentage values for the fatty acids EPA, AA, total *n*-6, the

omega 3 index (EPA+DHA) and n-6/n-3 PUFA were observed in the venous isolated erythrocyte samples in comparison to DBS. This was to be expected due to whole capillary blood containing plasma and a number of other cell types including monocytes. For example, the levels of *n*-3 PUFA fatty acids in PMBCs are lower than RBCs that have a slower turnover with a longer lifespan of approximately 120 days in the circulation (Katan et al, 1997). In addition, erythrocyte FA concentration is not affected by acute dietary changes, as would be the case with the serum fraction of a whole blood capillary sample (Katan et al, 1997). Even though erythrocyte fatty acid values were higher than DBS values, we still expected there would be a relationship between the methods. Regression analysis showed there was only a significant correlation for EPA. In addition, for the other fatty acids there was evidence of proportional bias suggesting the difference between methods varied throughout the range of measurement. This shows that although there were differences between the methods, the differences varied and lacked consistency. As a result of these findings, RBC isolation was adopted to determine the fatty acid status of participants in chapter 5.

There were a number of limitations to consider in chapter 4. If capillary and venous whole blood samples (rather than isolated RBCs) had been compared, there may have been a relationship between the methods. Alternatively it would have been interesting if isolated RBCs had been spotted onto a Guthrie card. This would have allowed determination of whether differences in the ratio of blood components between the two sampling methods were responsible for the lack of relationship, or if it was a result of the sample procedure and subsequent analysis. For example, the degradation of samples due to oxidation is a known limitation with DBS samples. Bailey-Hall et al. (2008) collected DBS on BHT treated filter paper in order to minimise fatty acid oxidation. However (Bell et al., 2011), found no significant difference with the use of blood collection paper pre-treated with BHT compared to

non-treated paper for analysis of fatty acids. AA, EPA and DHA percentage values reduced over a 4-wk storage period at room temperature from 2.93% to 1.95% (with most degradation occurring after 48 hours) the other fatty acid values reduced in a similar way. This suggests BHT may not necessarily improve sample stability.

In chapter 5, the aims of this thesis were two-fold. Firstly, to investigate the effect of a high *n*-3, low *n*-6 PUFA (L3-H6) dietary intervention, on isolated erythrocyte PUFA levels. Secondly, to examine the effect of this dietary intervention on oxygen consumption during submaximal exercise and $\dot{V}O_{2\text{ peak}}$ performance. It is apparent that modern diets have an unbalanced ratio of *n*-6 PUFA to *n*-3 PUFA of approximately 16:1, rather than 1:1. The latter is the ratio observed in the diet of wild animals and the presumed traditional ratio of fats required in the human diet (Eaton, 1985., Simopoulos, 1991). Increased *n*-3 PUFA intake results in changes in cell membrane phospholipid fatty acid composition. This may influence the physical properties of the cell membrane resulting in changes in cell signalling pathways, membrane receptors, gene expression and the production of inflammatory lipid mediators (Calder, 2006). *N*-3 and *n*-6 PUFA compete for the same elongase and desaturase enzymes for incorporation into cell phospholipids (Mickleborough, 2008). This raises the question of whether *n*-3 PUFA incorporation could be further augmented by reducing dietary *n*-6 LA intake. The majority of previous studies have adopted *n*-3 PUFA supplementation for a period of 3-10-weeks at approximate daily dosages of between 2 - 6.5g/ day of fish oil typically containing approximately 2g EPA and 1.5g DHA (Buckley et al., 2007; Walser et al., 2008; Da Boit et al., 2015; Kawabata, 2014; Gravia et al., 2017). A couple of studies have supplemented with higher DHA than EPA (Nieman et al., 2009; Hingley, 2017). Only one clinical study to date has reported methods to lower dietary *n*-6 PUFA, whilst maintaining or

increasing *n*-3 PUFA intake. Macintosh et al. (2013), investigated two dietary interventions, 1) average *n*-3, Low *n*-6 (L6) and 2) high *n*-3, low *n*-6 (H3-L6). In both interventions *n*-6 LA was lowered to $\leq 2.5\%$ of daily average energy intake. The L6 diet not only reduced *n*-6 PUFA but also increased *n*-3 PUFA EPA (+51%) and DHA (+19%) erythrocyte incorporation. This suggests that high *n*-6 PUFA diets may interfere with the incorporation of *n*-3 PUFA in human tissues. The H3-L6 diet markedly increased *n*-3 PUFA EPA and DHA and reduced *n*-6 PUFA erythrocyte incorporation when compared with the L6 diet. Based on these findings a novel H3-L6 dietary intervention was adopted in chapter 5 that reduced dietary *n*-6 PUFA to $\leq 2.5\%$ of average daily energy intake and enhanced *n*-3 PUFA fatty acid intake by supplementing with EPA (2600mg) and DHA (1800mg) daily for 8 weeks. The diet intervention successfully increased EPA (pre to post: 1.30 ± 0.20 to 5.85 ± 1.68 , $p < 0.001$), DHA (4.97 ± 2.82 to 9.35 ± 1.12 , $p = 0.001$) and total *n*-3 PUFA (10.43 ± 2.53 to 20.13 ± 2.83 $p < 0.001$). Furthermore, the intervention reduced LA (15.17 ± 1.92 , to 11.98 ± 1.30 $p = 0.004$), AA (19.99 ± 2.53 to 15.39 ± 1.19 $p < 0.001$) and total *n*-6 PUFA (40.27 ± 1.85 to 30.75 ± 1.29 $p < 0.001$). Interestingly, in the fish oil group, *n*-3 PUFA EPA and DHA increased and *n*-6 LA and AA reduced most markedly by week 4 of the supplementation period after which their levels plateaued apart from DHA, which took 8 weeks to significantly increase in concentration. DHA may be taken up by erythrocytes at a slower rate than EPA. An important methodological point in randomised controlled trials is consideration of the target tissue and its degree of *n*-3 PUFA uptake. An individual's fatty acid status is often measured in isolated erythrocytes as a surrogate marker of human tissue status. However it has been established that the myocardium and skeletal muscle tissue readily take up DHA preferentially to EPA (Herbst, 2013). Therefore, measurement of fatty acid status in erythrocytes may not be representative of the fatty acid status of all cell species.

Limitations

Fatty acid analysis confirmed that the participants had adhered to *n*-3 PUFA supplementation however, it is difficult to substantiate if the low *n*-6 PUFA diet had any additional effect on the lowering of LA, AA or total *n*-6 compared to *n*-3 PUFA supplementation alone (as we didn't employ an *n*-3 PUFA supplementation only group). Previous studies that report increases in *n*-3 PUFA through supplementation also observe decreases in *n*-6 PUFA without any background dietary manipulation. It is also difficult to compare the current study values to other reported values, due to the variety of supplementation protocols employed in previous research.

Rather than using food frequency questionnaires as employed by Macintosh et al., (2013), seven-day food records were analysed (Nutritics University edition). Food records are a more valid measure of nutrient intake however, they may be unsuitable for large studies or when sufficient instruction is not provided (Kolar et al, 2005). From participant's diet records, high *n*-6 PUFA foods were identified and substituted for low *n*-6 PUFA alternatives. The substituted 7-day diet was returned to the participants and they were asked to follow this dietary intake for the 8-week supplementation period. Even small changes to dietary intake can be a burden to participants, and this dietary intervention was highly reliant on subject compliance. Macintosh et al., (2013) mitigated this burden somewhat by providing meals for a 2-week dietary intervention, the participants also attended nutrition counselling sessions every 2 weeks. In the current study participants met with the researcher 4 weeks into the intervention period to discuss how the dietary intervention was progressing and if they needed any alternative meal/ snack suggestions. Convenient self-report measures such as 24-hour recall could have been implemented to encourage further participant compliance.

The effects of the novel H3-L6 dietary intervention on submaximal oxygen consumption and performance of $\dot{V}O_{2\text{ peak}}$ was investigated in chapter 5. It has been reported that human heart tissue readily incorporates *n*-3 PUFA DHA (Metcalf et al., 2007; Owen et al., 2004). Increased DHA in myocardial phospholipids has been shown to enhance ventricular function by reducing the oxygen requirement to produce any given work output, without affecting the work rate of the heart (Pepe & McLennan, 2002). Furthermore, Incorporation of *n*-3 PUFA, has been shown to reduce heart rate and improve the function of the heart both at rest (Mozzafarian et al, 2005) and when oxygen demand is elevated (McLennan, 2014). Previous studies have shown that FO supplementation lowers heart rate during submaximal exercise in healthy and trained participants (Buckley et al. 2009., Peoples et al, 2008) Peoples et al, (2008), reported that trained cyclists who supplemented with *n*-3 PUFA had lower heart rates during a $\dot{V}O_{2\text{ peak}}$ test and throughout ~60min submaximal exercise at sustained intensity to exhaustion. Furthermore, Peoples and McLennan (2008), observed reduced whole body oxygen consumption by skeletal muscle whilst muscular work did not change during sustained submaximal exercise at 55% $\dot{V}O_{2\text{ peak}}$ to exhaustion. Kawabata (2014), observed improved exercise efficiency during steady state exercise and a reduction in perceived exertion. Collectively these studies provide evidence that *n*-3 PUFA may modify oxygen utilization by the heart and skeletal muscle due to increased efficiency or enhanced blood flow. A number of studies have suggested that *n*-3 PUFA supplementation may augment blood flow and oxygen delivery to the exercising muscles via enhanced vasodilation, possibly through enhanced endothelial NO release (Walser et al., 2006; Zebrowska et al 2014). Therefore a main aim of this thesis was to investigate the effect of a novel H3 – L6 dietary intervention on oxygen consumption over 4 submaximal workloads (100 W, 125 W, 150 W, 175 W) similar to those used to determine the gross efficiency protocol (Passfield and Doust, 2000).

Previous research has often examined oxygen consumption during fixed intensity exercise (Peoples and McLennan, 2008). Walser and Stebbins (2008) employed a 10 minute cycling protocol at a low (90 – 100 bpm) and moderate (> 125 bpm) intensities. DHA and EPA supplementation augmented increases in stroke volume and cardiac output in the moderate exercise condition only. Therefore, a range of submaximal workloads was selected. Although the dietary intervention successfully achieved the target increase in *n*-3 PUFA and decrease in *n*-6 PUFA, it did not elicit changes in $\dot{V}O_{2\text{ peak}}$ performance or oxygen consumption during submaximal exercise at 4 submaximal work rates. No changes in local tissue oxygen saturation index (TSI %) or deoxy HHb were detected using NIRS analysis, furthermore there were no changes in HR or BP when compared to the control group. These findings are in contrast to those of Leaf and Rauch (1988), and Zebrowska et al (2014), who observed an increase in $VO_{2\text{ max}}$ in trained men. In addition, this study does not support the findings of previous research that observed reduced oxygen consumption during exercise following *n*-3 PUFA supplementation (Kawabata, 2014; Peoples and McLennan 2008, 2010; Hingley, 2017). The findings of the current study are in agreement with Oostenbrug et al. (1997), Raastad (1997), Buckley (2007) and Nieman et al. (2009), who failed to report any improvement in $\dot{V}O_{2\text{ max}}$ or associated physiological variables following *n*-3 PUFA supplementation. However, it should be considered there is large variation between studies regarding *n*-3 PUFA daily dosage and duration of supplementation as well as the type of exercise protocol employed (see table 2.4 in chapter 2).

Limitations

For the submaximal exercise test, absolute workloads were employed of 100, 125, 150 and 175 watts for 7 minutes. These are relatively low work rates for a short

duration of time. Peoples and McLennan (2008), employed a submaximal cycling protocol at a work rate of 55% of peak work load (approximately 240 watts taken from the mean pre supplementation W_{peak} figures) this is a higher intensity than the current study and participants exercised for a longer duration until exhaustion ~60 minutes. It is possible that, if the main n -3 PUFA mechanism is oxygen delivery, the current exercise protocol may not have been intense enough. The oxygen supply to meet demand was easily met under these conditions. An athlete may need to be in a state of fatigue or oxygen deficit to observe the possible cardiovascular compensatory effects of n -3 PUFA. Hingley, (2017) found there was no effect of DHA rich fish oil on oxygen consumption during low and moderate intensity exercise which agrees with previous findings (Gerling et al., 2014). However, reduced oxygen consumption was observed during a 5 minute cycling time trial when the physiological effort was much higher (See future recommendations).

Future research recommendations

High intensity aerobic exercise

A number of studies have begun to investigate the effects of n -3 PUFA on intense exercise performance. Hingley, (2017) examined the effect of n -3 PUFA on the performance of a time trial when athletes were in a fatigued state, theorising that the oxygen efficiency/ utilisation related mechanisms linked to n -3 PUFA might reveal themselves or compensate an athlete performing a time trial under such conditions. This was unique in comparison to previous research examining submaximal low intensity exercise. Hingley et al, (2017) reported that 8-weeks of n -3 PUFA supplementation, did not influence maximal cycling power (3 x 6 seconds), or repeated Wingate cycling (6 x 30 seconds with 150 seconds active recovery). There

was no observed effect of supplementation on time trial performance (5 minutes cycling as hard and as fast as possible in a fatigued state) or isometric strength of the quadriceps (determined by three maximal voluntary quadriceps contractions). However, mean oxygen consumption relative to workload was significantly lower over the 5 minute cycling time trial, with most notable reduction occurring during the first 2 minutes of exercise performance, when participants were under severe physiological strain. This suggests that the *n*-3 PUFA, in particular DHA, is important for skeletal muscle function during high intensity activity. No changes were observed in the *n*-3 PUFA group during the moderate 10-minute steady state exercise test. It should be noted this study used a low dose of FO compared to previous studies. 2g/day fish oil of which 140mg was EPA and 560mg DHA. Follow up studies would be interesting that examine DHA and supra- maximal constant load cycling or a protocol that will allow the separation of peripheral and centrally induced fatigue mechanisms. It would also be interesting to examine *n*-3 PUFA and its effect in conditions of oxygen deprivation, where oxygen delivery to the muscle might be compromised such as, exercise in hypoxic conditions or in individuals with exercise induced asthma. In addition, it is important for future research to question if the potential *n*-3 PUFA mechanisms (related to lowered $\dot{V}O_2$ cost) are due to *n*-3 PUFA affecting the delivery of oxygen to the skeletal muscle via improvements in blood flow or due to improved mechanical efficiency of the heart and skeletal and muscle respiratory mechanics.

N-3 PUFA and post exercise immune recovery

A further area of research related to *n*-3 PUFA that requires more examination is the benefit of *n*-3 PUFA's in the post exercise recovery period when the immune system can be compromised affecting recovery time (Walsh et al, 2011; Nieman &

Mimesser, 2017). It is well documented that dietary enrichment of *n*-3 PUFA reduces the production of arachidonic acid-derived (AA) eicosanoid mediators due to reduced levels of AA and competitive incorporation of *n*-3 PUFA FA in the desaturation elongation cascade. In addition, it appears that long-term *n*-3 PUFA supplementation alters the EPA and DHA membrane composition of immune cells, influencing factors such as phagocytosis. Altered gene expression increases the number of phagocyte surface receptors increasing their uptake of target material Calder, (2003). A 6-month supplementation period of 1.5g EPA and DHA increased neutrophil and monocyte phagocytic activity by approximately 40% and 200% respectively (Kew, et al, 2003).

In addition, It has been established that *n*-3 PUFA's induce the production of inflammation resolving mediators called E series resolvins (from EPA) and maresins, D series resolvins and protectins (from DHA) (Nieman & Mimesser, 2017). Resolvins, protectins and maresins decrease the production of PGE₂, and pro-inflammatory cytokines (Yaqoob, 1995). These pro-resolving mediators help regulate and resolve acute inflammation (Kelley et al., 1998). High intensity cycling for 75-km, resulted in post exercise elevation in most inflammatory mediators that reduced back to baseline levels by the following morning. Maresin 1 was elevated during this recovery process, which may suggest its importance in recovery from inflammation (Nieman, 2017).

The research conducted to date establishes the importance of immune cell membrane fatty acid composition and the influence of *n*-3 PUFAs on cell function. However many immune function studies are carried out in-vitro. Further research is required to investigate the effect of *n*-3 PUFA on inflammatory mediators over chronic periods of training. Few studies have examined the effect of fish oil on post exercise recovery of immune function and the evidence available is conflicting,

mainly due to differences in research designs. The research field is lacking large studies in athletic groups.

n-3 PUFA and strength speed and power exercise

It is clear that previous exercise and training studies have focused on n-3 PUFA and its therapeutic effect on cardiovascular function. Few studies have assessed *n-3* PUFA in relation to muscle function and the performance of strength, speed and power based exercise, which are also important components of intermittent team based sport performance. In studies of this nature, it is important to consider the diverse requirements of team sport performance with a multifactorial exercise testing approach. Recently Gravina et al., (2017) examined a 4 week supplementation intervention with high dose *n-3* PUFA 0.1g/kg per day on adaptations in strength, speed, power and anaerobic endurance capacity in International soccer players. The results demonstrated a potential benefit of *n-3* PUFA on anaerobic endurance capacity in a yo yo intermittent test, where more distance was covered following the training and supplementation intervention this was theorised to be due possibly to improved blood flow to skeletal muscle which may have prolonged time to fatigue. However, there were no observed improvements in strength, power or speed assessments.

n-3 PUFA and skeletal muscle metabolism

n-3 PUFA supplementation and its effect on human skeletal muscle metabolism is an area of contemporary investigation especially with regard to muscle protein synthesis. There is evidence to suggest that *n-3* PUFA intake positively affects strength gains following resistance training in the elderly (Smith et al, 2011). There

may be an anabolic effect of *n*-3 PUFA supplementation, the mechanisms mediated by increased activation of mTOR - p70 ribosomal protein S6 kinase (p70S6K) signalling (this signal can be blunted in older age due to increased inflammation). McGlory et al. (2014) investigated the effect of *n*-3 PUFA supplementation for a period of 8-weeks on myofibrillar protein synthesis (MPS). In addition, the influence of *n*-3 PUFA combined with protein feeding and exercise on MPS was examined. Fish oil supplementation did not influence myofibrillar protein synthesis rate at rest, in combination with protein feeding or with protein feeding post exercise. Reduced PKB and p70S6K1 activity was observed 3 hours post exercise suggesting that *n*-3 PUFA supplementation might influence anabolic signalling without any change in MPS. This area warrants further research.

CONCLUSIONS & CONTRIBUTION TO THE FIELD

Firstly, this thesis examined the acute cytokine response to two novel time trial and interval cycling protocols. This thesis contributes knowledge to the field that both a 1-hour time trial and an interval cycling trial elicit an increase in IL-6, and the increase was more marked in the time trial compared to the interval trial. This finding is typical of the frequently reported IL-6 response to exercise. In addition, anti-inflammatory IL-4 and MCP-1 increased and VEGF decreased post exercise. Neither protocol response supports the traditional view that IL-6 production triggers the acute production of pro-inflammatory TNF α or IL-1 β (Peake, 2005). The findings were however in agreement with more recent research that has failed to detect changes or only slight increases in pro-inflammatory cytokines post 90 minutes running, eccentric and exhaustive exercise (Ostapiuk-Karolczuk et al., 2012; Hirose et al., 2004 & Suzuki et al., 2002). Therefore, cycling protocols of this nature do not appear to be acutely inflammatory, although a delayed increase in the appearance

of inflammatory cytokines could not be ruled out in this study due to the limited sampling time points post exercise. Additional blood sample collections in the hours and days post exercise would provide further knowledge regarding cytokine kinetics in response to different cycling protocols.

Due to the potential influence of *n*-3 PUFA on cardiovascular variables, this thesis investigated its effect on oxygen consumption and cardiovascular variables during cycling exercise. This research question presents a number of methodological considerations regarding the relevant daily dosage of *n*-3 PUFA and the duration of supplementation. It is important for studies to be able to accurately quantify the extent of *n*-3 PUFA tissue incorporation resulting from a supplementation protocol using rapid convenient methods. Therefore, this thesis examined the relationship between rapid fingertip capillary DBS and gold standard isolated erythrocyte methods of measurement to quantify an individual's fatty acid status. The study was novel as it correlated samples from 3 time points over an 8 week supplementation period (Baseline, 4 weeks and at 8 weeks) whereas previous research has examined levels at rest (without supplementation) (Bailey-Hall et al., 2008). This study provides evidence that the rapid DBS method is suitable for the measurement of EPA only, but not other fatty acids. Isolated erythrocyte analysis is the most valid method of measurement for studies that need to quantify the incorporation of multiple PUFAs or over a prolonged supplementation protocol; therefore this method of analysis was adopted in chapter 5.

In relation to the "ideal dose" and dose duration of *n*-3 PUFA, previous studies have employed a variety of different protocols. However, given that *n*-3 and *n*-6 PUFA compete for the same desaturase elongation enzyme for incorporation into phospholipid membranes, it was relevant to consider lowering background dietary *n*-6 PUFA intake whilst supplementing with *n*-3 PUFA. Furthermore, to examine dose response over an 8-week supplementation protocol, fatty acid incorporation

was measured at baseline, 4 weeks and 8 weeks into the supplementation period. Previous studies have only considered pre and post measures. A novel dietary intervention that lowered *n*-6 and increased *n*-3 PUFA dietary intake through supplementation with 2600mg EPA and 1800mg DHA/ day of fish oil successfully increased erythrocyte membrane levels of EPA and DHA and reduced *n*-6 AA and LA. Although the dietary intervention was successful in achieving its target *n*-3 and *n*-6 PUFA levels, it did not reduce oxygen consumption during steady state submaximal exercise or associated cardiovascular variables. In addition, there was no change in $\dot{V}O_{2\text{ peak}}$ performance following the dietary intervention over 4 submaximal cycling intensities. This work contributes to the field in a number of ways. Previous studies have measured oxygen consumption data using gas exchange methods (Douglas bag or online breath-by-breath systems). This was the first study to combine gas exchange methods with local muscle oxygenation (TSI %) and oxygen extraction (deoxy HHb) using NIRS analysis. Local measurements in the current thesis appeared to compliment the findings at the gas exchange level i.e. no change in $\dot{V}O_2$ consumption, TSI % or deoxy HHb. It is suggested that future work should focus on the influence of *n*-3 PUFA on skeletal muscle respiration and mitochondrial bioenergetics at higher workloads where oxygen delivery is compromised to see if the possible compensatory cardiovascular effects of *n*-3 PUFA reveal themselves during physiologically stressful exercise.

In short. This thesis has contributed new investigative work regarding exercise induced inflammation and the influence of *n*-3 PUFAs on oxygen consumption and cardiovascular variables. The findings suggest *n*-3 PUFA does not reduce oxygen consumption during submaximal cycling or influence the performance of $\dot{V}O_{2\text{ peak}}$. The work in this thesis has uncovered a number of important methodological considerations regarding the limited use of DBS methods. In addition, a novel and

successful dietary model has been created that considers the modulation of background dietary *n*-6 PUFA intake. These findings are important for future research design. Whilst *n*-3 PUFA has been recommended to athletic populations, based on the findings of some of the exercise literature discussed in this thesis. It cannot be concluded from these findings if such a recommendation is warranted. There is still much research to uncover regarding the mechanisms of *n*-3PUFA in the athletic context.

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APPENDIX

APPENDIX 1

CHAPTER 4

The following procedures for fatty acid analysis (Chapter 4) were carried out by the analytical chemistry department at Loughborough University

Reagent and biological materials

Reagents that were used in preparing samples include; acetyl chloride, potassium carbonate (K_2CO_3), and butylated hydroxyl toluene (BHT). HPLC grade n-hexane, methanol and heptadecanoic acid methyl ester was used as internal standard along with 9 ml Ace pressure tubes were all purchased from Sigma Aldrich. Supelco 37 Mix FAME standard (Sigma Aldrich) was used as the certified reference material for GC-MS analysis of complex mixture of saturated, monounsaturated and polyunsaturated fatty acids. 50 μ g/ml BHT-methanol was prepared and added to all samples to prevent oxidation of fatty acids

Sample preparation

Samples were removed from the -80 °C freezer, thawed and vortexed to ensure sample homogenization, 100 μ l of the sample (RBCs or PBMCs) was added to the reaction tube containing the reaction medium which was composed of 3.4 ml of 50 μ g/ml BHT in methanol and 200 μ L of acetyl chloride and sealed. BHT was not added to the reaction medium used for DBS analysis as Guthrie cards were treated with BHT prior to sampling. For DBS samples, one of the 30 mm² punched samples was derivatised by adding 200 μ L of acetyl chloride to 3.4 ml of methanol.

The reaction medium of BHT/ Methanol /Acetyl chloride was freshly prepared in the reaction tubes while the sample was thawed. The reaction was heated at 70 °C for 60 minutes in a water bath and 5 ml of 6% (K₂CO₃) was added to stop and neutralize the reaction. 1.5 ml of n-hexane was added and the mixture was centrifuged at 2500 rpm for 10 minutes after which the top hexane layer was taken. The washing step was repeated and the combined hexane layers were evaporated to dryness using nitrogen gas. Samples were reconstituted in 100 µl of hexane containing 10 µg/ml of internal standard. 1 µl aliquot of sample was then injected on the GC-MS by a CTC Analytics Combi-Pal auto sampler.

Analysis of Fatty acids

Fatty acid composition of RBC, DBS and PBMCs was determined by gas chromatography (GC) mass spectrometry (MS). The GC oven temperature program began at 130 °C and increased from 130 °C to 208 °C at a rate of 6 °C /min. The temperature program then increased from 208 °C at 2 °C /min until it reached 225 °C where it was held for 10 min with a total analysis time of 31.5 min. Injector temperature was set at 220 °C with a split ratio of 10:1. Helium was used as carrier gas at constant flow rate of 1.5 ml/min. 1 µl aliquot of fatty acid methyl ester (FAME) sample was injected on the GC by a CTC Analytics Combipal auto sampler. FAMES were characterized by electron ionization (EI) in positive ion full scan mode.

APPENDIX 2

CHAPTER 5

Two way mixed ANOVA analysis summary of total saturated fat and monounsaturated fat

Total Saturated fat

A two way mixed ANOVA elicited no significant main effect of time, $F(2, 26) = 1.98$, $p = 0.16$, partial $\eta^2 = 0.13$ or group, $F(1, 13) = 0.051$, $p = 0.825$, partial $\eta^2 = 0.004$. There was no significant interaction between time and group, $F(2, 26) = 1.251$, $p = 0.30$, partial $\eta^2 = 0.88$, on erythrocyte total saturated fat content.

Total Monounsaturated fat

A two way mixed ANOVA elicited no significant main effect of time, $F(2, 26) = 1.625$, $p = 0.22$, partial $\eta^2 = 0.11$ or group $F(1, 13) = 0.548$, $p = 0.472$, partial $\eta^2 = 0.04$. There was no significant interaction between time and group, $F(2, 26) = 3.03$, $p = 0.06$ partial $\eta^2 = 0.18$, on erythrocyte total monounsaturated fat content.

APPENDIX 3

CHAPTER 5

Three way mixed ANOVA analysis summary of RPE, lactate and glucose changes over the four - stage power test between groups, pre to post supplementation with n-3 PUFA.

RPE

There was a significant main effect of power, $F(1.541, 20.038) = 162.49$, $p < 0.001$, partial $\eta^2 = 0.92$. There was no significant main effect of pre/ post, $F(1,13) = 0.009$, $p = 0.93$, partial $\eta^2 = 0.001$, or group, $F(1, 13) = 0.001$, $p = 0.98$, partial $\eta^2 = <0.001$. There was no statistically significant two-way interaction between power & group, $F(1.541, 20.038) = 2.97$, $p = 0.085$, partial $\eta^2 = 0.19$, between pre/post and group $F(1, 13) = 0.45$, $p = 0.84$, partial $\eta^2 = 0.003$, or pre/post and power $F(3, 39) = 0.506$, $p = 0.68$, partial $\eta^2 = 0.037$. Furthermore there was no significant three way interaction between group, pre/post and power level, $F(3, 39) = 0.14$, $p = 0.94$, partial $\eta^2 = 0.01$.

Table 18. RPE values pre and post the supplementation period in the FO and SO group at each power level

		Pre	Post	N
100 W	FO	8.9 ± 1.5	9.1 ± 2.19	7
	SO	8.4 ± 1.3	8.4 ± 1.7	8
125 W	FO	10.4 ± 1.9	10.4 ± 2.07	7
	SO	10.4 ± 1.5	10.5 ± 1.6	8
150 W	FO	11.4 ± 1.5	11.4 ± 1.62	7
	SO	11.8 ± 1.3	11.6 ± 1.4	8
175 W	FO	12.6 ± 1.1	12.4 ± 1.13	7
	SO	13.1 ± 0.9	12.8 ± 1.1	8

Note: Data are expressed as means ± SD

Whole blood lactate levels (mmol/L) during the four stage submaximal test (power level).

There was a significant main effect of power, $F(4, 52) = 5.229$, $p = 0.001$, partial $\eta^2 = 0.29$. There was no significant main effect of pre/ post, $F(1, 13) = 0.198$, $p = 0.66$, partial $\eta^2 = 0.02$ or group, $F(1, 13) = 2.08$, $p = 0.17$, partial $\eta^2 = 0.138$. There was no significant two-way interaction between power level & group, $F(4, 52) = 1.14$, $p = 0.35$, partial $\eta^2 = 0.08$, pre/post and group $F(1, 13) = 0.19$, $p = 0.66$, partial $\eta^2 = 0.015$, or pre/post and power, $F(1.759, 22.87) = 0.84$, $p = 0.43$, partial $\eta^2 = 0.06$. Furthermore there was no significant three way interaction between power level, pre/post and group $F(1.759, 22.87) = 0.84$, $p = 0.43$, partial $\eta^2 = 0.06$ (Tables 21 and 22).

Glucose (BG mmol/L) during the four stage submaximal test (power level).

There was a significant main effect of power, $F(1.529, 19.877) = 6.748$, $p = 0.009$, partial $\eta^2 = 0.342$. There was no significant main effect of pre/ post, $F(1,13) = 0.217$, $p = 0.65$, partial $\eta^2 = 0.16$ or group, $F(1,13) = 0.219$, $p = 0.65$, $\eta^2 = 0.17$.

There was no statistically significant two-way interaction between power level & group, $F(1.53, 19.88) = 2.46$, $p = 0.057$, partial $\eta^2 = 0.159$ or between pre/post and group $F(1, 13) = 0.005$, $p = 0.94$, partial $\eta^2 = <0.001$. There was a significant two way interaction between pre/post and power level $F(1.76, 22.93) = 4.12$, $p = 0.006$, partial $\eta^2 = 0.24$. Furthermore, there was no significant three way interaction between power level, pre/post and group $F(1.764, 22.93) = 2.27$, $p = 0.13$, partial $\eta^2 = 0.15$ (Tables 23 & 24).

Table 21. Lactate (mmol/L) pre and post the supplementation period in the FO and SO group at each power level

		Pre	Post	N
Baseline	FO	1.69± 0.39	1.70± 0.35	7
	SO	1.87±0.50	1.59±0.72	8
100 W	FO	1.81± 0.67	2.21± 0.73	7
	SO	2.11± 0.61	2.39±1.41	8
125 W	FO	1.53± 0.56	1.49± 0.49	7
	SO	2.05± 0.58	2.06± 0.67	8
150 W	FO	2.09± 0.76	1.78± 0.48	7
	SO	2.17± 0.61	2.04± 0.51	8
175 W	FO	2.64± 1.44	2.03± 0.53	7
	SO	2.81± 0.70	2.33± 0.65	8

MAXIMAL OXYGEN UPTAKE TEST

RPE

A 3 way mixed ANOVA was performed to examine any interaction between groups pre and post the supplementation period (pre/post), on RPE values each minute of the $\dot{V}O_2$ max test (time). The first 10 minutes of the test was included in the analysis.

There was a significant main effect of time $F(1.567, 17.24) = 172.80, p = <0.001$, partial $\eta^2 = 0.94$. There was no significant effect of pre/ post, $F(1, 11) = 0.041, p = 0.84$, partial $\eta^2 = 0.004$ or group, $F(1, 11) = 0.199, p = 0.664$, partial $\eta^2 = 0.018$.

There was no statistically significant two-way interaction between pre/post and group $F(1, 11) = 4.186, p = 0.065$, partial $\eta^2 = <0.28$, between time & group, $F(1.57, 17.24) = 1.249, p = 0.30$, partial $\eta^2 = 0.10$ or pre/post and time $F(3.73, 40.99) = 0.191, p = 0.093$, partial $\eta^2 = 0.017$. Furthermore, there was no statistically significant three way interaction between time, pre/ post and group $F(3.73, 40.99) = 1.32, p = 0.28$, partial $\eta^2 = 0.11$.

Lactate (Post $\dot{V}O_{2\max}$ test)

A two-way mixed ANOVA was performed to examine any interaction between group and whole blood lactate levels following the completion of the $\dot{V}O_{2\max}$ test pre and post the supplementation period. There was a significant main effect of time (pre/ post) $F(1, 13) = 10.99, p = 0.006$ partial $\eta^2 = 0.458$, and no significant main effect of group on whole blood lactate levels, $F(1, 13) = 0.014, p = 0.907$ partial $\eta^2 = 0.001$. Furthermore, there was no statistically significant two way interaction between group and time (pre/ post), $F(1, 13) = 0.21, p = 0.65$ partial $\eta^2 = 0.016$.

A paired samples *t*-test indicated that blood lactate was significantly higher post the $\dot{V}O_{2\max}$ compared to pre (as to be expected). Post value (mean \pm stdev) = 8.9 mmol/ L \pm 2.86), pre value = 10.74 mmol/ L \pm 2.35. A statistically significant mean increase of 1.83, 95% CI [0.69 to 2.96], $p = 0.004$.

Post $\dot{V}O_2$ max Glucose

A 2 way mixed ANOVA was performed to examine any interaction between the intervention group SO/FO and post max test whole blood glucose levels pre and post the supplementation period. There was no significant main effect of time (pre or post the supplementation period) $F(1, 13) = 1.908$, $p = 0.190$ partial $\eta^2 = 0.128$, and no significant main effect of group $F(1, 13) = 6.41$, $p = 0.025$ partial $\eta^2 = 0.33$. Furthermore, there was no significant two way interaction between the group and pre/ post $F(1, 13) = 1.989$, $p = 0.182$ partial $\eta^2 = 0.133$.